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TITLE: SLC5A8-Mediated Switching of STAT3 from a Pro-Oncogenic Signal into a Pro-Apoptotic Signal in Breast Cancer


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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Overall our findings demonstrated that the novel tumor suppressor SLC5A8 is necessary to mediate the pro-apoptotic function of STAT3 in the normal mammary epithelial cells. To support our findings, we observed that the functional inactivation of SLC5A8 is associated with mammary gland involution delay, hyperplasia, and early onset of mammary tumorigenesis as well as accelerated lung metastasis. SLC5A8 associated tumor suppression is mainly linked to its effects of transporting the physiological substrate, butyrate, in to the mammary epithelium. Interestingly, mammary gland specific Slc5a8 expression (MMTV-Slc5a8-Tg) is associated with precocious mammary gland involution and significant protection against MMTV-Neu-Tg and MMTV-HRas-Tg induced mammary tumor and its associated lung metastasis. Further, STAT3 and SLC5A8 are necessary for the milk stasis induced cellular apoptosis in mammary epithelium. Functional inactivation of either Stat3 or Slc5a8, using the Stat3 and Slc5a8 siRNAs, efficiently blocks milk stasis induced apoptosis in mammary epithelial cells. In addition, continuous release of butyrate (90 days release) provokes mammary gland involution by activating the apoptotic signaling in the mouse mammary glands. Over all our results provide in vivo evidence that functional Slc5a8 is necessary to mediate Stat3 induced apoptosis in mammary epithelium as well as to prevent the Stat3 associated mammary tumorigenesis.

**15. SUBJECT TERMS**
Mammary tumor, Stat3, Slc5a8, MMTV-Slc5a8-Tg, MMTV-Neu-Tg, MMTV-HRas-Tg

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# Table of Contents

**INTRODUCTION:** Breast cancer is a disease driven by progressive genetic abnormalities, which include mutations in tumor suppressor genes or oncogenes, and epigenetic events such as DNA methylation and histone deacetylation. In the process of mammary tumorigenesis, several tumor suppressor genes are either inactivated or down-regulated with constitutive activation of several oncogenes. However, certain genes play dual role in the process of tumorigenesis. For example, TGFβ acts as a tumor suppressor, inhibit cell proliferation and induce apoptosis in tumor cells during the early stages of tumor formation; however, during later stages of tumorigenesis, tumor cells become unresponsive to the growth inhibitory actions of TGFβ and become more motile, more invasive, and more resistant to apoptosis (1-3). Likewise, STAT3 plays a prominent role in cell death associated with post-lactational regression of the mammary gland through the classical as well as non-classical cell death signaling pathways (4, 5). Further, conditional deletion of *STAT3* is associated with delayed mammary gland involution (6). STAT3 is phosphorylated and consequently activated in mammary epithelial cells during mammary gland involution and this activation is accompanied with a differential change in the expression of the regulatory subunits of PI3 kinase (PI3K): a decrease in p85α and an increase in p55α and p50α. Interestingly, all three subunits are coded by a single gene, *pik3r1*, using different promoters (7, 8). Studies by Abell *et al* (9) have shown that the promoter specific for p55α and p50α are direct targets for activated STAT3. However, mammary gland involution is associated not only with an increase in the expression of these two subunits but also with a decrease in the expression of p85α. In addition, STAT3 activation is also associated with induction of growth arrest and apoptosis in mouse mammary epithelial cells, monocytes, myeloid cells and granulocytes and also involved in maintenance of pluripotency of embryonic stem cells (10-13). Conversely, STAT3 has also been designated as a proto-oncogene; it is constitutively activated in a wide variety of tumors, including breast cancer (14, 15). The activated STAT3 is associated with cell survival and proliferation, inhibition of apoptosis, activation of angiogenesis and suppression of immune surveillance (16). Based on these findings, STAT3 is generally considered as a growth-promoting factor. However, recent studies have shown that STAT3 functions as either tumor suppressor or proto-oncogene depending on the mutational profile of the tumor (17). STAT3 inhibits astrocyte proliferation and invasiveness and suppresses the malignant transformation, which is associated with PTEN inactivation. Further, loss of PTEN triggers the down-regulation of the cytokine receptor LIFRβ (Leukemia Inhibitory Factor Receptor β) in astrocytes and consequently inhibits the phosphorylation of STAT3 at the key regulatory site Tyr705. In human glioblastoma specimens, PTEN loss correlates tightly with low levels of LIFRβ expression and inactivation of STAT3. In contrast, STAT3 forms a physical complex with the pro-oncogenic protein EGFRvIII (Epidermal Growth Factor Receptor type III variant) and mediates EGFRvIII-induced astrocyte transformation (17). Thus, depending on the binding partner, STAT3 functions as either tumor-suppressor or proto-oncogene. However, the molecular switch that dictates the tumor-suppressive or tumor-promoting signaling of STAT3 has not been identified.

*SLC5A8*: SLC5A8 is a plasma membrane transporter, which was originally identified as a putative tumor suppressor in colon cancer (18), but neither the identity of its transport function nor how it elicits tumor suppression was known. Studies from our laboratory have not only established the identity of the transport function of this protein but also the mechanism by which its transport function promotes tumor cell-specific apoptosis. Further, SLC5A8 is not only silenced in colon cancer but it is silenced in more than 10 different types of cancers including breast cancer. This is the first tumor suppressor ever identified in a very broad spectrum of human malignancies. The tumor suppressor function of SLC5A8 is mainly associated with inhibition of histone deacetylases (HDACs) in the tumor cells. SLC5A8 is a $Na^+$-coupled transporter for short-chain fatty acids (e.g., propionate, butyrate) and other monocarboxylates (e.g., lactate, pyruvate) (19, 20). Butyrate is a well-known HDAC inhibitor, and HDAC inhibitors preferentially kill cancer cells without affecting the normal cells (21-25). Butyrate is generated at high concentrations in colonic lumen by bacterial fermentation of dietary fiber, and SLC5A8 is expressed in the lumen-facing apical membrane of

colonic epithelial cells, mediating the entry of butyrate into cells (24, 25). This provides a molecular mechanism for the transporter's role as a tumor suppressor in colon. However, the expression of SLC5A8 is down-regulated in tumors of various non-colonic tissues, but how this transporter elicits a tumor-suppressive effect on non-colonic tissues was not known. Attempts in our laboratory to answer this question led to the discovery that pyruvate, an endogenous metabolite and a substrate for SLC5A8, is a potent inhibitor of HDACs and an inducer of tumor cell-specific apoptosis (21). These findings explain not only why SLC5A8 is down-regulated in many tumors, but also why tumor cells effectively convert pyruvate into lactate. Lactate is also a substrate for SLC5A8, but it does not inhibit HDACs. In order to avoid the entry of the HDAC inhibitors, pyruvate and butyrate, tumor cells intentionally down-regulate the expression of SLC5A8 to escape from cell death (21). Further, it has long been known that accumulation of milk during mammary gland involution induces apoptosis in mammary epithelium, but the underlining molecular mechanisms were not known. With studies completed using the recent DOD idea award, we have delineated that STAT3 and SLC5A8 are the molecular mediators of milk stasis-induced apoptotic signaling. Breast milk contains substantial amounts of butyrate in the form of triglycerides, which releases free butyrate in mammary ducts upon milk stasis. The milk stasis activates STAT3 through stretching, and this activated STAT3, in turn, induces SLC5A8; then SLC5A8 transports butyrate into mammary epithelial cells, which then initiates apoptosis by HDAC inhibition. Mammary gland involution is always associated with coordinated STAT3 activation and SLC5A8 induction. In contrast, breast cancer cells possess constitutively active STAT3, but *SLC5A8* is silenced by DNA methylation. Thus, when the expression of SLC5A8 is normal, activated STAT3 is associated with induction of apoptosis, maintains the cellular homeostasis and functions as a tumor suppressor; in the absence of SLC5A8, however, activated STAT3 is associated with cell survival, inhibits apoptosis and functions as a proto-oncogene. Based on these observations, *we hypothesize that SLC5A8 is the molecular controller of STAT3-mediated pro-apoptotic signal responsible for mammary gland involution and mammary tumor prevention*. This is a novel and innovative idea in the area of breast cancer and provides a molecular basis for the tumor-suppressive role of SLC5A8 and a rational explanation as to why this gene is silenced in breast cancer. Testing of this hypothesis is the primary goal of this project. If this hypothesis turns out to be correct, it will have far-reaching implications in the treatment of breast cancer, making SLC5A8 as a novel therapeutic target. Pharmacologic means to re-activate *SLC5A8* expression in breast cancer cells will then have potential to convert activated STAT3 in these cells from a pro-oncogenic signal into a pro-apoptotic signal.

**BODY:** Development of stable cell lines and related characterization like analyzing the gene expression level and cell cycle responses have shown in the attached conference abstracts and posters (please see the attachment in the bottom). Other than these in vitro studies, we are giving the in vivo studies using the SLC5A8 wild type and knockout, mammary gland specific SLC5A8 expressing transgenic mice and its role in the mammary gland development and mammary tumorigenesis are shown below. We are preparing two manuscripts based on the data obtained from this grant proposal, which are intended to submit one to "Cell" and to "Cancer Cell". We will forward the manuscripts once it will be accepted.

*Stat3 and Slc5a8 are co-operatively regulated during mammary gland involution*: We and others have shown that Stat3 is activated during early onset of mammary gland involution and that conditional deletion of *Stat3* is associated with delaying of mammary gland involution (6, 29). We have also shown that knockdown of C/ebpδ, a down-stream target of Stat3, results in a similar phenotype such as delayed mammary gland involution and flawed apoptotic regulation in the mammary epithelium (29). In addition, we have shown that *Slc5a8* is a transcriptional target of C/ebpδ (30). However, whether or not the expression of Slc5a8 is modulated during the mammary gland involution was not known. Therefore, we analyzed the expression of Slc5a8 in virgin

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mammary gland, lactating mammary gland, and also during different times of mammary gland involution. These studies showed that Slc5a8 expression is dramatically induced during the early onset of mammary gland involution. Further, Slc5a8 is specifically localized in the apical membrane of the mammary ducts (Fig. 1A and B). As a positive control, we analyzed the expression of Stat3 and C/ebpδ (Fig. 1C and D).

**Fig. 1: Slc5a8 expression is induced during early onset of mammary gland involution**. Mammary glands [virgin (V), lactation day 10 (involution day 0) and different times of involution (12-96 h)] were collected from the normal C57BL/6 mice, and RNA and protein samples were prepared. Tissue sections were also prepared for immunohistochemical (IHC) analysis. **(A)** Slc5a8 mRNA expression was analyzed by semi-quantitative and real-time PCR using mouse Slc5a8-specific primers. **(B)** Slc5a8 protein expression (western blot) and localization (IHC) were analyzed using an anti-Slc5a8 antibody (**Red**). We used CD98 as a marker for the basolateral membrane (**Green**) and DAPI (**Blue**) for nuclear staining. **(C) & (D)** Stat3 and C/ebpδ mRNA expressions were analyzed by semi-quantitative PCR using mouse Stat3- and C/ebpδ-specific primers. We also analyzed the phosphorylated (p-Stat3), acetylated (Ac-Stat3) and total Stat3 as well as C/ebpδ protein using specific antibodies.
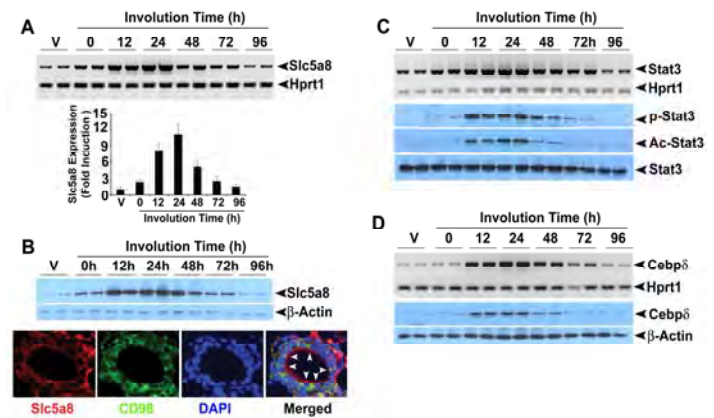


Figure 1

*Slc5a8 knockout delays mammary gland involution*: Like Stat3, Slc5a8 expression is also induced during the early onset of mammary gland involution. However, the functional importance of Slc5a8 in the regulation of mammary gland involution is not known. Therefore, we wanted to delineate the role of Slc5a8 in mouse mammary gland involution. For this, we analyzed the morphological changes in the mammary gland in wild type (*Slc5a8*$^{+/+}$) and *Slc5a8*-knockout (*Slc5a8*$^{-/-}$) mice by H & E staining (Fig.1B and C), mammary epithelial apoptosis by Apoptag staining and various apoptosis markers (data not shown) and expression of several mammary gland involution markers such as Stat3 (Fig.1A). We also analyzed the whole mount of Scl5a8$^{+/+}$ and Slc5a8$^{-/-}$ mouse mammary gland (Fig.1D). We found that the deletion of *Slc5a8* is associated with mammary gland involution delay. Wild type alveoli are almost completely collapsed (panels C & E); in contrast, the alveoli in *Slc5a8*-null glands are still intact (panels D & F) at the involution day 3 and 4. We also quantified the content of fat cells, a marker for mammary gland involution, in wild type and *Slc5a8*$^{-/-}$ mammary glands (Fig. 2C). Further, the whole mount analysis showed that deletion of *Slc5a8* is associated mammary gland hyperplasia (Fig. 2D). All these results suggest that functional Slc5a8 is necessary to mediate the apoptosis signaling in the post-lactational mammary gland and to maintain the cellular homeostasis in the mammary epithelium.

**Fig. 2: Deletion of *Slc5a8* delays mammary gland involution and induces mammary gland hyperplasia**. Mammary glands (wild type and *Slc5a8*$^{-/-}$) were collected from virgin (Vir) mice and at different times of involution. RNA, protein, tissue sections and whole mounts were prepared. **(A)** Slc5a8 and Stat3 expressions were analyzed by semi-quantitative RT-PCR. **(B)** H & E staining of the sections from virgin (A-B), involution days 3 (C-D) and 4 (E-F). **(C)** Percent of fat cells during mammary gland involution was quantified using the NIH1.62 image software. Values are shown as a mean $\pm$ SEM of 12 different regions of 3 animals. **(D)** Ductal and terminal end bud density determined using the whole mounts of mammary glands, demonstrating ductal hyperplasia in *Slc5a8*$^{-/-}$ mice.
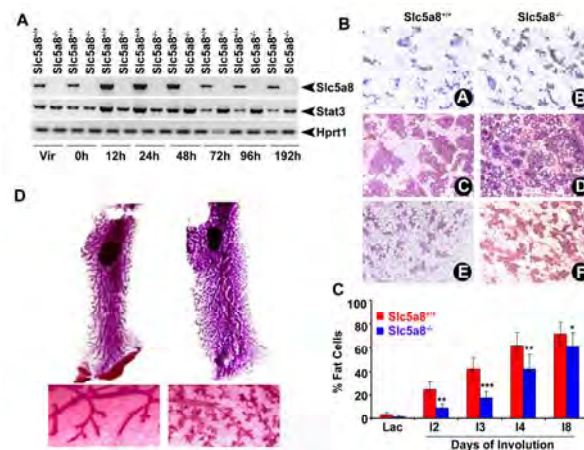


Figure 2

**Functional Slc5a8 is necessary for butyrate-induced HDAC inhibition in mammary epithelium:** The tumor suppressor function of SLC5A8 is associated with inhibition of HDACs and the substrates of SLC5A8 (butyrate and pyruvate) are HDAC inhibitors. Therefore, we determined the role Slc5a8 in transporting the HDAC inhibitor butyrate into the mammary epithelium to cause HDAC inhibition. We focused on butyrate for two reasons: (a) Milk stasis that signals mammary gland involution results in accumulation of butyrate in the ductal lumen; (b) Slc5a8, the transporter for butyrate, is localized to the lumen-facing apical membrane of the ductal epithelial cells. We prepared nuclear extracts from the mammary glands (wild type and *Slc5a8*-null) of virgin mice, pregnant mice, lactating mice and at different times of involution and measured the HDAC activity using a commercially available HDAC assay kit (BioVision). *Slc5a8*-null mice showed relatively high HDAC activity than wild type mice at all time points examined (Fig. 3A). Since there are several isoforms of HDACs, we evaluated the specificity of butyrate inhibition. We used the commercially available human recombinant HDACs (HDAC1, 2, 3, 5, 6, 8 and HDAC9) for these studies. Butyrate (1 mM) specifically inhibits HDAC1 and HDAC3 and has no effect on other isoforms (Fig. 3B). Interestingly, during mammary gland involution, both HDAC1 and HDAC3 expressions are significantly reduced in wild type mammary gland, but this was not the case in *Slc5a8*-null mammary glands, indicating that in wild type mice HDAC activity is reduced during involution not only because of Slc5a8-mediated entry of butyrate but also because of the decreased expression of HDAC1 and HDAC3. Thus, Slc5a8 is obligatory to transport butyrate into the mammary epithelium. Further, since milk stasis causes enlargement of the ductal lumen, thus causing stretching of the epithelial cells, we asked whether stretching influences the expression of Stat3 and Slc5a8 in these cells. We found that mechanical stretching of mammary epithelial cells (HC11, a mouse mammary epithelial cell line) does indeed induce the expression of these two genes and also causes HDAC inhibition in the presence of butyrate (Fig. 3C). To examine the relevance of Slc5a8 in the transport of butyrate, we isolated primary mammary epithelial cells from the one-day post-involuting mammary glands of wild type and *Slc5a8*-null mice and then incubated these cells in the presence and absence of butyrate and subjected to mechanical stretching. Our results again confirmed that stretching inhibits HDACs and induces apoptosis only in the presence of Slc5a8 and butyrate (Fig. 3D and E). Butyrate does not produce these effects in *Slc5a8*$^{-/-}$ cells.



**Fig. 3: Slc5a8 is obligatory for butyrate-induced HDAC inhibition. (A)** Nuclear extracts were prepared from the mammary glands of *Slc5a8*$^{+/+}$ and *Slc5a8*$^{-/-}$ virgin (V), pregnant day 10 (P10), lactation day 10 (L10) and different times of involution (I0.5, I1, I2, I3, I4 and I8) and HDAC activity was measured. **(B)** Activities of human recombinant HDACs (HDA1, 2, 3, 5, 6, 8 and HDAC9) were monitored in the absence and presence of 1mM butyrate. **(C)** Normal mouse mammary epithelial cell line, HC11, was cultured in the presence and absence of butyrate for 24 h and then subjected to mechanical stretching for 3 h. Nuclear extracts were prepared and HDAC assay was measured. **(D & E)** Primary mammary epithelial cells were prepared from the *Slc5a8*$^{+/+}$ and *Slc5a8*$^{-/-}$ mice mammary glands (involution day 1) and cultured in the presence and absence of butyrate for 24 h and then subjected to mechanical stretching. Part of the cells was used to extract the nuclear fraction, which was used to measure HDAC activity **(D)**. The remaining cells were used for the cell cycle analysis **(E)**. Values are given as mean ± SEM of three independent experiments.
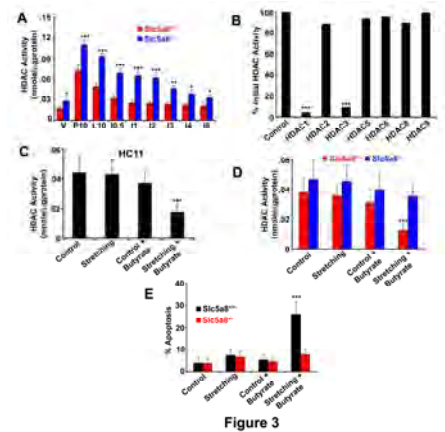
**Butyrate implantation provokes early onset of mammary gland involution:** Our results show that milk stasis/stretching induces Stat3 and Slc5a8, and that Slc5a8 transports butyrate from the milk and induces apoptosis during mammary gland involution. To confirm these observations further, we implanted butyrate tablets for a slow and continuous release of butyrate (90 days release) in *Slc5a8*$^{+/+}$ and *Slc5a8*$^{-/-}$ mice. Mammary gland morphology was analyzed in control and the butyrate-implanted mice. Our results show a precocious mammary gland involution in butyrate-implanted wild type (*Slc5a8*$^{+/+}$) mice (Fig. 4A; panels A-F). But, butyrate has no effect in *Slc5a8*-

null mice (Fig. 4A; panels G-L). These results clearly demonstrate functional obligation Slc5a8 to transport butyrate into the mammary ductal epithelium and, in turn, this transported butyrate induce HDAC inhibition and induce apoptosis and thereby it induces mammary gland involution.



**Fig. 4: Butyrate implantation provokes early onset of mammary gland involution.** A slow and continuous release of butyrate (10 mg for 90 days release) was implanted into $Slc5a8^{+/+}$ and $Slc5a8^{-/-}$ mice. Mammary glands were collected from virgin mice and involution days 2 and 3 mice. **(A)** Sections of virgin (A-B; G-H), involution day 2 (C-D; I-J) and involution day 3 (E-F; K-L) mammary glands were analyzed for the morphological changes. **(B)** Fat cells contents were quantified using the NIH1.62 image program and values are shown as mean $\pm$ SEM of 12 sections each from 3 mice.
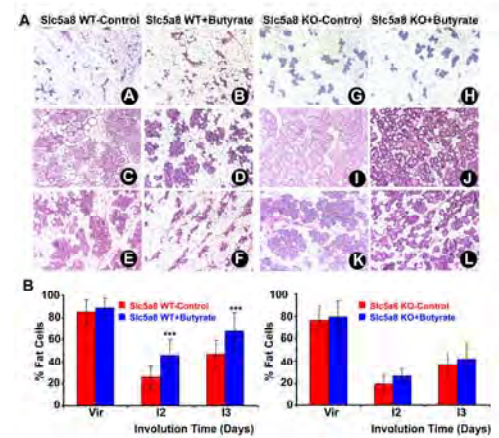
**Mammary gland-specific Slc5a8 over-expression accelerates mammary gland involution:** Since the endogenous Slc5a8 plays an essential role in apoptosis associated with mammary gland involution via its ability to mediate the entry of the HDAC inhibitor butyrate into mammary epithelial cells, we hypothesized that over-expression of Slc5a8 in mammary epithelial cells would accelerate the rate of mammary gland involution. To test this hypothesis, we developed a mouse with mammary gland-specific over-expression of Slc5a8 using the MMTV promoter. As predicted, these mice showed accelerated mammary gland involution (Fig. 5) and accelerated pro-apoptotic signaling (data not shown). These results clearly demonstrate the obligatory role of Slc5a8 in regulation of mammary gland involution.



**Fig. 5: Mammary gland-specific over-expression of Slc5a8 in mice provokes mammary gland involution and triggers apoptotic signaling.** Mammary glands were collected from control C57BL/6 mice and from MMTV-Slc5a8-Tg mice (virgin and involution days 2, 3 and 4). **(A)** Mammary gland alveoli status, whether it is collapsed or intact, was monitored and the representative images are shown (Panels A-H). **(B)** We also quantified the fat cell contents using the NIH1.62 image software. Values are expressed as mean $\pm$ SEM of three 3 mice in each group.
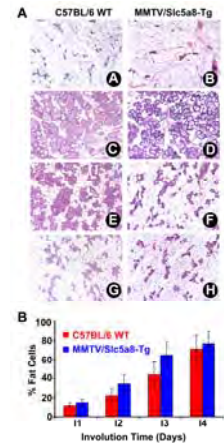
**Deletion of $Slc5a8$ is associated with early onset of mammary tumorigenesis and enhanced lung metastasis:** Our results suggest that Slc5a8 is necessary for the proper restructuring of the mammary gland after weaning via induction of apoptosis in mammary epithelial cells and that deletion of $Slc5a8$ is associated with mammary gland involution delay and hyperplasia. Based on these data, we hypothesized that deletion of $Slc5a8$ inactivation would be associated with early onset of mammary tumorigenesis. To test this hypothesis, we crossed the $Slc5a8$-null mouse with MMTV-Neu-Tg mouse and generated all three genotypes ($Slc5a8^{+/+}$, $Slc5a8^{+/-}$ and $Slc5a8^{-/-}$) with MMTV-Neu-Tg background. Our preliminary results showed that deletion of $Slc5a8$ is associated with high incidence of mammary tumor (Fig. 6A, 64% vs. 90% for $Slc5a8^{+/+}$ and $Slc5a8^{-/-}$, respectively), early onset of mammary tumorigenesis (Fig. 6B, 315 days vs. 201 days for $Slc5a8^{+/+}$ and $Slc5a8^{-/-}$, respectively), increased number of tumors (Fig. 6C), and accelerated lung metastasis (Fig. 6D). These preliminary results suggest that Slc5a8 is not only involved in mammary gland involution but also protects the mammary epithelial cells from the cellular transformation and tumorigenesis, thus functioning essentially as a tumor suppressor.

**Fig. 6: Deletion of $Slc5a8$ is associated with early onset of mammary tumorigenesis.** $Slc5a8$-null female mouse was cross-bred with MMTV-Neu-Tg male mouse to generate $Slc5a8^{+/+}$-MMTV-Neu, $Slc5a8^{+/-}$-MMTV-Neu and $Slc5a8^{-/-}$-MMTV-Neu mice. The rate of tumor incidence **(A)**, time of tumor appearance (B), number of tumors per mouse **(C)** and incidence of lung metastasis **(D)** were monitored. Values are shown as mean $\pm$ SEM.

**Mammary gland-specific over-expression of Slc5a8 protects against mammary tumorigenesis:**
If Slc5a8 does indeed function as a tumor suppressor in mammary gland as suggested from our studies, over-expression of this protein should protect against mammary tumorigenesis. To test this, we crossed MMTV-Slc5a8-Tg mice with MMTV-Hras-Tg mouse, and analyzed the rate and time of tumor incidence, number of tumor per mouse, rate of lung metastasis, tumor size and disease-free survival time. Our results showed that over-expression of Slc5a8 in mammary gland effectively prevents mammary tumorigenesis as evident from the decreased tumor incidence (Fig. 7A, 75% vs. 41% of control MMTV-Hras and MMTV-*Slc5a8*/MMTV-Hras mouse, respectively), delayed tumor formation (Fig. 7B. 318 days vs. 352 days of control MMTV-Hras and MMTV-*Slc5a8*/MMTV-Hras mouse, respectively), reduced numbers of tumor (7C), reduced lung metastasis (7D), reduced tumor size (7E), and increased disease-free survival (7F).



**Fig. 7: Mammary gland-specific over-expression of Slc5a8 protects against mammary tumorigenesis.** Using the control MMTV-Hras mice and MMTV-Hras/MMTV-*Slc5a8* mice, we monitored the rate of tumor incidence **(A)**, time of tumor incidence **(B)**, number of tumors in each mouse **(C)**, lung metastasis **(D)**, tumor size **(E)** and average survival time **(F)**. Values are expressed as mean ± SEM.
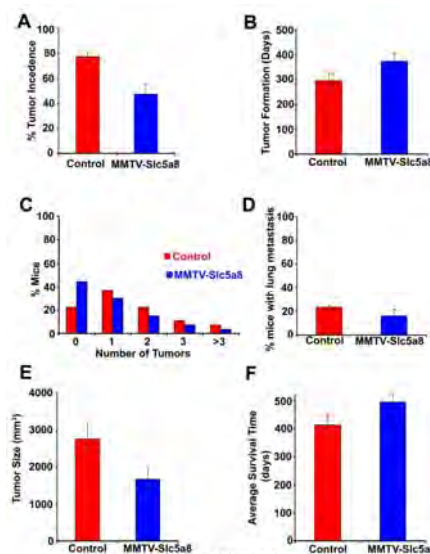
In summary, our results have shown that Stat3 and Slc5a8 are coordinately regulated during mammary gland involution and that deletion of Slc5a8 delays mammary gland involution and induce mammary gland hyperplasia. Further, deletion of Slc5a8 significantly reduced the Stat3 expression, delayed mammary gland involution, and enhanced ductal proliferation (Refer Fig. 2a). Also, continuous release of butyrate accelerates mammary gland involution and mammary gland-specific over-expression of Slc5a8 provokes early onset of mammary gland involution. In addition, deletion of Slc5a8 is associated with early onset of mammary tumorigenesis and enhanced lung metastasis. Mammary gland-specific over-expression of Slc5a8 efficiently protects the mouse against mammary tumorigenesis. Thus, in the normal mammary epithelium, milk stasis activates Stat3 and, in turn, this activated Stat3 induces Slc5a8 expression; then Slc5a8 transports butyrate into the mammary epithelium, which then inhibits HDAC1 and 3 and triggers apoptotic signaling cascade. Inactivation of either Stat3 or Slc5a8 delays mammary gland involution by defective regulation of apoptotic signaling pathway. However, in the mammary tumor, Stat3 is constitutively active while Slc5a8 is inactive; thus, in the absence of Slc5a8, Stat3 binds to the oncogenic signaling molecules such as EGFR, HRAS, etc and switches into the oncogenic mode rather than the tumor suppressor mode.

KEY RESEARCH ACCOMPLISHMENTS:

- Analyzed the expression pattern of Slc5a8 and Stat3 during mouse mammary gland development.
- Analyzed the acetylation status of Stat3 during mouse mammary gland development.
- Analyzed the expression pattern of SLC5A8 in human normal and breast cancer cells.
- Analyzed the acetylation and phosphorylation status of Stat3 in human normal and breast cancer cells.
- Developed all stable cell lines, which express constitutively active STAT3C in normal mammary epithelial cell line as well as in human breast cancer lines and analyzed the Slc5a8 expression, activity and also analyzed DNA methyl transferases (DNMTs) expression and activity. Using these cell lines, we also analyzed histone deacetylases (HDACs) expression and activity.

- Analyzed the mammary gland development pattern using the Slc5a8 wild type (Slc5a8$^{+/+}$) knock out (Slc5a8$^{-/-}$) and found that Slc5a8 knockout is associated with involution delay, mammary gland hyperplasia with reduced expression of Stat3.
- Slc5a8 inactivation is also associated with increased HDAC activity.
- Analyzed the functional significance of Stat3 and Slc5a8 in milk stasis induced apoptosis using the stable expression of Stat3siRNA and Slc5a8siRNA in mouse and human normal mammary epithelial cell lines.
- Crossed Slc5a8 knockout mice with two mouse mammary tumor models (MMTV-Neu-Tg and MMTV-HRas-Tg) and developed Slc5a8$^{+/+}$-MMTV-Neu-Tg and Slc5a8$^{-/-}$-MMTV-Neu-Tg as well as Slc5a8$^{+/+}$-MMTV-HRas-Tg and Slc5a8$^{-/-}$-MMTV-HRas-Tg mice.
- Slc5a8 inactivation is associated with early onset of mammary tumorigenesis and provokes lung metastasis of mammary tumor and significantly reduced the disease free survival.
- Developed the Slc5a8-MMTV-Tg and analyzed the role of Slc5a8 in mammary tumor prevention and or protection using the two mouse mammary tumor models (MMTV-Neu-Tg and MMTV-HRas-Tg).
- Mammary gland specific expression of Slc5a8 protects the MMTV-Neu and MMTV-HRas induced mammary tumorigenesis and increase the disease free survival.

REPORTABLE OUTCOMES:

**1.Manuscripts**:

We are in the process of submitting two manuscripts, which are intended to submit to "Cell" and to "Cancer Cell".  We will forward these manuscripts once it will be accepted.

On manuscript, which is not directly related to the work we proposed in this grant, however, the training and experience that obtained from this grant, has been accepted in the Cancer Research and we gave acknowledged our generous grant agency of CDMRP (Please find an attachment for the acceptance letter and copy of the manuscript)

**2. Abstracts:**

(1) We submitted an abstract in the 101st AACR conference entitled "**Differential Regulation of STAT3 in the Involution and in Tumorigenesis of the Mammary Gland**" (See the attached abstract and poster**).**

(2) We also submitted an abstract in the 2011 Era of Hope meeting entitled "**SLC5A8  is a molecular mediator of STAT3 associated pro-oncogenic signal into pro-apoptotic signaling in breast cancer**" (See the attached abstract and poster**).**

(3) We also submitted an abstract in the AACR meeting of New Horizons in Cancer Research entitled "**Molecular mechanism of SLC5A8 inactivation in breast cancer**" (See the attached abstract).

**3. Presentations:**

Have presented the research findings, which related to this grant proposal in the 2011 Georgia Cancer Summit meeting, which was held in June 16-17 in Macon, GA entitled **"Slc5a8 inactivation predisposes to early onset of mammary tumorigenesis and provokes lung metastasis of breast cancer"**

**4. Patents and licenses applied for and/or issued:**

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There are no patents or licenses applied related to the proposed
work.
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**5. Degrees obtained that are supported by this award:**

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There are no degrees or diploma obtained by using this award.
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**6. Development of cell lines, tissue or serum repositories:**

We have developed the following were eight stable cell lines: HMEC, MCF10A and MCF7 cells with stable expression of constitutively active STAT3 (STAT3C), MCF7 and MB231 cells with Tet-On regulated lentiviral mediated stable expression of SLC5A8, Stable expression of SLC5A8siRNA in two normal mammary epithelial cell lines (HMEC and MCF10A) as well as stable expression of STAT3shRNA in MCF7-SLC5A8 stable cell line. We did not submit any repositories yet.

**7. Informatics such as databases and animal models, etc.:**

We have developed three animal models using this grant:

(1) We have generated mammary gland specific Slc5a8 expressing transgenic mice using the mouse mammary tumor virus (MMTV) promoter (MMTV-Slc5a8-Tg)

(2) We have crossed this MMTV-Slc5a8-Tg mouse with the mouse mammary tumor model mice MMTV-Neu-Tg and generated MMTV-Slc5a8/MMTV-Neu-Tg mouse.

(3) Similarly, we have also crossed this MMTV-Slc5a8-Tg mouse with another mouse mammary tumor model mouse and MMTV-HRas and generated MMTV-Slc5a8/MMTV-HRas-Tg mouse.

**8. Funding applied for based on work supported by this award:**

Based on the result that we obtained from this grant, we have submitted an application for Department of Defense (DOD) Idea Expansion Award recently.

**9. Employment or research opportunities applied for and/or received based on experience/ training supported by this award:**

No employment or research opportunities applied so far but in the future, after the publication of our finding that obtained from this grant proposal, should be useful for the promotion and the better job opportunities.

CONCLUSIONS:

The main conclusion of our study is that:
(1) STAT3 and SLC5A8 are cooperatively expressed an regulated in the normal mammary epithelium

(2) STAT3 functions as a pro-apoptotic and cellular homeostasis regulator in the presence of functional SLC5A8 in the normal mammary epithelium.

(3) Similarly, in the mammary tumorigenesis, even though STAT3 is constitutively active, the functional SLC5A8 overcome the pro-oncogenic potential of STAT3.

(4) Slc5a8 inactivation is associated with mammary gland involution delay, hyperplasia, and predispose early onset of mammary tumorigenesis and provokes lung metastasis.

(5) <u>Both STAT3 and SLC5A8 knockout showed the similar phenotype, like mammary gland involution delay, mastitis and hyperplasia. However, our present study shows that SLC5A8 inactivation is associated to early onset of mammary tumorigenesis and accelerates lung metastasis and it is not known whether mammary gland specific conditional STAT3 knockout mouse showed the similar phenotype, like SLC5A8, or STAT3 inactivation protect the mammary tumorigenesis. This needs to be done in the future studies</u>.

(6) Milk stasis induced apoptosis is initiated by STAT3 in the normal mammary epithelial cells and SLC5A8 mediates the STAT3 initiated apoptotic signaling.

(7) Functional inactivation of either STAT3 or SLC5a8 abolished the milk stasis induced apoptosis and these results showed that both STAT3 and SLC5A8 are the major components of the milk stasis induced apoptosis.

(8) SLC5A8 associated tumor suppression is mainly linked to its transport capacity of its physiological substrate, butyrate, into the mammary epithelial cells.

(9) Constitutively active Slc5a8 in the mammary epithelium efficiently protect and prevent the mammary tumorigenesis, even though STAT3 is constitutively active in this mouse mammary tumor model.

(10) Functional reactivation of SLC5A8 in the mammary tumorigenesis will be the effective treatment strategy for the breast cancer management.

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APPENDICES:

(1) Abstract of 101st AACR conference
(2) Poster of 101st AACR conference
(3) Abstract of 2011 Era of Hope conference
(4) Poster of 2011 Era of Hope conference
(5) Abstract of AACR meeting of New Horizons in Cancer Research.


BINDING:   Because  all  reports  are  entered  into  the  Department  of  Defense
Technical Reports database collection and are microfiched, it is recommended that
all  reports  be  bound  by  stapling  the  pages  together  in  the  upper  left  hand
corner.    All  original  reports  shall  be  legible  and  contain  original
photos/illustrations.  Figures shall include figure legends and be clearly marked
with figure numbers.

# Differential Regulation of STAT3 in the Involution and Tumorigenesis of Mammary Gland

Sabarish Ramachandran, Selvakumar Elangovan, Puttur D. Prasad, Vadivel Ganapathy and
Muthusamy Thangaraju
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STAT3 (Signal Transducer and Activator of Transcription 3) is persistently activated in many human primary tumors as well as in cancer cell lines, including breast cancer. This activated STAT3 is associated with cell survival and proliferation, inhibition of apoptosis, activation of angiogenesis and suppression of immune surveillance. Thus, STAT3 is generally considered as a growth-promoting factor. However, STAT3 activation also plays an important role in initiation of apoptotic signaling, especially, in mammary epithelial cells during mammary gland involution. Conditional deletion of STAT3 is associated with delayed involution mainly due to inadequate induction of apoptosis in mammary epithelial cells. Additionally, STAT3 activation is associated with induction of growth arrest and apoptosis in mouse mammary epithelial cells, myeloid cells and granulocyte. Thus, STAT3 plays dual role as a growth promoter as well as a growth inhibitor. However, the molecular switch that is responsible for switching the STAT3-associated pro-oncogenic signal to pro-apoptotic signal is not known. In order to identify this molecular switch we screened for signaling molecules that regulate STAT3 as well as those that are regulated by STAT3 during mammary gland involution, in human primary breast tumor tissues, and in human breast cancer cell lines. Our results show that early in the onset of mammary gland involution there is a significant upregulation of STAT3 and SLC5A8, a $Na^+$-coupled transporter for monocarboxylates, including butyrate an inhibitor of histone deacetylases (HDACs). Further, STAT3 expression during mammary gland involution was found to be predominantly acetylated and phosphorylated. However, in human breast cancer cell lines we found an increase in phosphorylated STAT3 with almost undetectable expression of SLC5A8 and significantly decreased expression of acetylated STAT3 expression. Heterologous expression of SLC5A8 in human mammary tumor cell lines resulted in a butyrate-dependent induction of apoptosis with an increase in acetylated forms of STAT3. These results suggest that SLC5A8 is the molecular switch that converts the pro-oncogenic STAT3 signal to a pro-apoptotic signal by mediating the butyrate entry into mammary epithelial cells and thus it activates death receptor signaling by inhibiting HDACs and activation of STAT3 acetylation during mammary gland involution. However, in cancer cells the expression of SLC5A8 is silenced and thus it no longer able to inhibits HDACs and activates Ac-Stat3 and death receptor signaling. Thus, our studies provide a molecular link between the STAT3-associated pro-apoptotic and pro-oncogenic signaling that observed in normal mammary epithelium and in human breast cancer, respectively.

Short title: Pro-apoptotic to pro-oncogenic switch of STAT3 in mammary epithelium

# Differential Regulation of STAT3 in the Involution and in Tumorigenesis of the Mammary Gland

**Sabarish Ramachandran**, Selvakumar Elangovan, Vaishali Handa, Puttur D. Prasad, Vadivel Ganapathy and Muthusamy Thangaraju

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101st ANNUAL MEETING 2010
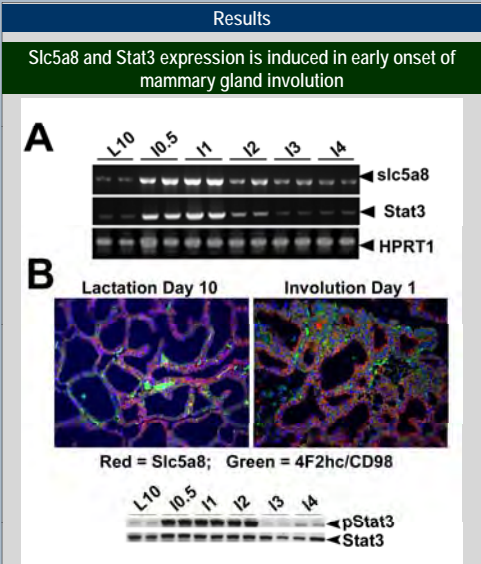April 17 - 21, 2010
Washington, DC

## Abstract

STAT3 (Signal Transducer and Activator of Transcription 3) is persistently activated in many human primary tumors as well as in cancer cell lines, including breast cancer. This activated STAT3 is associated with cell survival and proliferation, inhibition of apoptosis, activation of angiogenesis and suppression of immune surveillance. Thus, STAT3 is generally considered as a growth-promoting factor. However, STAT3 activation plays an important role in initiation of apoptotic signaling, especially, in mammary epithelial cells during mammary gland involution. Conditional deletion of STAT3 is associated with delayed involution mainly due to inadequate induction of apoptosis in mammary epithelial cells. Further, STAT3 activation is associated with induction of growth arrest and apoptosis in mouse mammary epithelial cells, myeloid cells and granulocyte. Thus, STAT3 plays dual role in growth inhibition and activation. However, the signaling molecule that is responsible for the STAT3 associated pro-apoptotic to pro-oncogenic signaling is not known. In order to understand the dual role of STAT3, we screened signaling molecules, which involved in regulation of STAT3 as well as the signaling molecules that are regulated by STAT3, during mammary gland involution and in human primary breast tumor tissues as well as in human breast cancer cell lines. Our results suggest that SLC5A8, a Na$^+$-coupled transporter for butyrate and other monocarboxylates as well as an inhibitor of histone deacetylases (HDACs), associated STAT3 acetylation (Ac-STAT3) play a crucial role in initiation and regulation of apoptotic signaling during the mammary gland involution. Both SLC5A8 and STAT3 expressions are highly upregulated in early onset of mammary gland involution and this enhanced STAT3 expression is mainly associated with the phosphorylated as well as acetylated form of STAT3. SLC5A8 play a crucial role in activation of Ac-STAT3 during mammary gland involution. However, the functional link between SLC5A8 and STAT3 acetylation that observed during mammary gland involution is missing in human primary breast tumor tissue as well as in human breast cancer cells. These results provide a molecular link between the STAT3 associated pro-apoptotic and pro-oncogenic signaling that observed in normal mammary epithelium and in human breast cancer, respectively.

## Background

There is unequivocal evidence for an oncogenic role of activated STAT3. Activation of STAT3 occurs via phosphorylation, mediated by classic growth-promoting signals. Furthermore, STAT3 is constitutively active in a variety of cancers. Therefore, activated STAT3 is widely considered as an oncogene with multiple roles related to cancer, including promotion of cell survival and proliferation, and suppression of immune surveillance. In contrast to what happens in cancer, activated STAT3 causes apoptosis in normal mammary epithelial cells during involution. This is evidenced from the suppression of epithelial cell apoptosis and delayed mammary gland involution in mice with conditional knockout of Stat3. The molecular mechanism responsible for the switching of activated Stat3 from a pro-oncogenic signal to a pro-apoptotic signal during mammary gland involution is not well understood. We recently fond SLC5A8, a Na$^+$-coupled monocarboxylate transporter and tumor suppressor, could be the molecular switch that convert the pro-oncogenic function of Stat3 into pro-apoptotic signaling. Our studies have shown that SLC5A8 mediated butyrate play a major role in Stat3 associated cellular apoptosis during mammary gland involution as well in human breast cancer cells.

## Objectives

**The objective of this study is to delineate the molecular switch, which converts the STAT3 associated pro-oncogenic signaling into pro-apoptotic and tumor suppressor function in human breast cancer.**

## Results

### Slc5a8 and Stat3 expression is induced in early onset of mammary gland involution



**Figure 1: Compromised expression of Slc5a8 and Stat3 in early onset of mammary gland involution. RNA and protein samples were isolated from the mammary glands of lactation (Day 10) and different time of involution (Day 0.5, 1, 2, 3 and 4). (A) Slac5a8 and Stat3 mRNA expression was analyzed by semi-quantitative PCR analysis using specific primers. (B) Slc5a8 protein expression was analyzed by immunohistochemical analysis by using Slc5a8 antibody and CD98 was used as a marker for basolateral membrane localization. Stat3 protein expression was analyzed by western blot analysis using phospho specific and total Stat3 antibodies.**

### Early onset of mammary gland involution is associated with inhibition of HDAC1 and HDAC3 and induction of Histone Acetylation



**Figure 2: Mammary gland involution is associated with inhibition of HDACs and activation of Histone Acetylation. RNA and protein samples were prepared from the mammary glands of Vir, lactation and different time of involution. (A) mRNA expression of histone deacetylases (HDACs) 1, 2, 3, 4, and 5 were analyzed by semi-quantitative PCR analysis using specific primers. (B) protein expression HDAC1, 2, 3, 4 and 5 was analyzed by western blot analysis using specific antibodies. (C) protein samples were prepared from the Slc5a8 WT (+/+) and Slc5a8 KO (-/-) mice at lactation day 10 and the involution day 0.5, 1 and 4. Expression of acetylated histone 3 (Ac-H3), acetylated histone 4 (Ac-H4), Ac-Lys12, Ac-Lys16, histone H3 and histone H4 were analyzed by western blot analysis using the specific antibodies.**
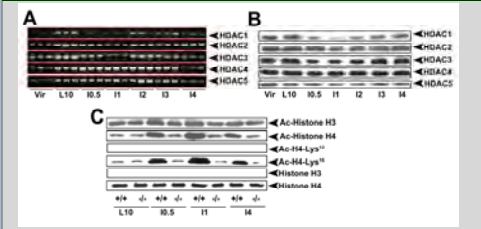
### Mammary gland involution is associated with activation of TRAIL and TRAIL receptor



**Figure 3: Mammary gland involution induced TRAIL and TRAIL receptor. TRAIL and TRAIL receptor expression were tested in the mammary glands of Virgin, lactation and different times of involution by Semi-quantitative PCR using the mouse TRAIL and TRAIL receptor specific primers.**

### Early onset of mammary gland involution is associated with acetylation and phosphorylation of Stat3



**Figure 4: Acetylation of Stat3 during mammary gland involution. Protein lysates were prepared from Virgin (Vir), pregnant days 10 and 15 (p10 and p15), lactation days 0, 5 and 10 (L0, L5 and L10) and involution days 0.5, 1, 2, 3, 4 and 5 (I0.5, I1, I2, I3, I4 and I5) and acetylation and phosphorylation status of Stat3 was analyzed by Western blot analysis. Two animals per time point were used in this experiment.**

### Mechanical stretching induces Slc5a8 and Stat3 acetylation and apoptosis in the presence of Slc5a8 substrates



**Figure 5: Mechanical Stretching induced Slc5a8 and Stat3 expression. RNA and protein were extracted from the HC11, a normal mouse mammary epithelial, cells that were mechanically stretched for 3h. (A) Slc5a8 and Stat3 mRNA expression was analyzed by semi-quantitative PCR. (B) Acetylation and phosphorylation status of Stat3 were tested by western blot analysis using specific antibodies. (C) Cellular apoptosis was measured in HC11 cells with and without mechanical stretching and Slc5a8 substrates pyruvate and butyrate by FACS analysis**
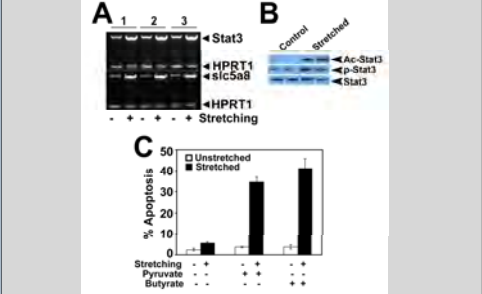
### Slc5a8 expression is associated with Stat3 acetylation in human mammary epithelial cells



**Figure 6: SLC5A8 expression in human mammary epithelial cells is co-relates with STAT3 acetylation in these cells. RNA and protein were extracted from the normal human mammary epithelial (HMEC, HBL100 and MCF10A) and human breast cancer (MCF7, T47D, ZR75.1, BT474, BT20, MB231, MB361, MB453, MB468 and HCC1937) cells. SLC5A8 mRNA expression was analyzed by semi-quantitative PCR and using a specific primers and acetylated and phosphorylated STAT3 were analyzed by western blot analysis using acetylation and phosphorylation specific STAT3 antibodies. Total STAT3 was used as an internal control.**

## Summary and Conclusions

- Slc5a8 and Stat3 expression are highly induced in early onset of mammary gland involution.

- Slc5a8 induction is associated with HDAC1 and HDAC3 down regulation during mammary gland involution.

- Slc5a8 and Stat3 activation is associated with TRAIL and TRAIL receptor activation during the early onset of mammary gland involution.

- Stat3 activation is associated with acetylation and phosphorylation of Stat3 in early onset of mammary gland involution.

- Mechanical stretching also associated with activation of Slcta8 and Stat3 acetylation and phosphorylation.

- Mechanical stretching induced mammary epithelial apoptosis only in the presence of Slc5a8 substrates pyruvate and butyrate.

- A compromised expression of Slc5a8 and Ac-STAT3 in human normal mammary epithelial cells. However, this link is missing in human breast cancer cells.

- Human breast cancer cells lost both Slc5a8 and STAT3 acetylation.

## Funding Support

# SLC5A8  is a molecular mediator of STAT3 associated pro-oncogenic signal into pro-apoptotic signaling in breast cancer

Sabarish Ramachandran, Selvakumar Elangovan, Puttur D. Prasad, Vadivel Ganapathy and

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# SLC5A8 is a molecular mediator of STAT3 associated pro-oncogenic signal into pro-apoptotic signaling in breast cancer

Sabarish Ramachandran, Selvakumar Elangovan, Puttur D. Prasad, Vadivel Ganapathy and

**Muthusamy Thangaraju**

Department of Biochemistry and Molecular Biology, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA - 30912

August 2-5, 2011
Orlando, FL

## Abstract

STAT3 (Signal Transducer and Activator of Transcription 3) is persistently activated in many human primary tumors as well as in cancer cell lines, including breast cancer. This activated STAT3 is associated with cell survival and proliferation, inhibition of apoptosis, activation of angiogenesis and suppression of immune surveillance. Thus, STAT3 is generally considered as a growth-promoting factor. However, STAT3 activation also plays an important role in initiation of apoptotic signaling, especially, in mammary epithelial cells during mammary gland involution. Conditional deletion of STAT3 is associated with delayed mammary gland involution mainly due to inadequate induction of apoptosis in mammary epithelial cells. Additionally, STAT3 activation is associated with induction of growth arrest and apoptosis in mouse mammary epithelial cells, myeloid cells and granulocyte. Thus, STAT3 plays dual role as a growth promoter as well as a growth inhibitor. However, the molecular switch that is responsible for switching the STAT3-associated pro-oncogenic signal into pro-apoptotic signal is not known. In order to identify this molecular switch, we screened signaling molecules that are regulated by STAT3 during mammary gland involution and in human normal and breast cancer cells. Our results showed that SLC5A8, a $Na^+$-coupled transporter for monocarboxylates and HDACs inhibitor, is co-operatively expressed with STAT3 during mammary gland involution. However, there is a reciprocal relationship between STAT3 and SLC5A8 expression in human breast cancer cells. Further, Slc5a8 knockdown is associated with mammary gland involution delay, same phenotype like Stat3 knockdown. Our preliminary results showed that Slc5a8 inactivation is associated with early onset of mammary tumorigenesis when crossed with two mouse mammary tumor models, MMTV-Neu-Tg as well as MMTV-Hras-Tg mice. In addition to that, functional activation of SLC5A8 in human breast cancer cells induced apoptosis and efficiently reduced the colony formation, which is associated with the constitutive STAT3 activation. SLC5A8-induced apoptosis and tumor suppression is associated with HDAC inhibition as well as the activation of death receptor signaling. Thus, our results suggest that SLC5A8 is a molecular dictator of STAT3-associated pro-apoptotic and pro-oncogenic signaling that observed in normal mammary epithelium and in human breast cancer, respectively.

## Background

There is unequivocal evidence for an oncogenic role of activated STAT3. Activation of STAT3 occurs via phosphorylation, mediated by classic growth-promoting signals. Furthermore, STAT3 is constitutively active in a variety of cancers. Therefore, activated STAT3 is widely considered as an oncogene with multiple roles related to cancer, including promotion of cell survival and proliferation, and suppression of immune surveillance. In contrast to what happens in cancer, activated STAT3 causes apoptosis in normal mammary epithelial cells during involution. This is evidenced from the suppression of epithelial cell apoptosis and delayed mammary gland involution in mice with conditional knockout of Stat3. Recent studies have also shown that Stat3 control lysosomal mediated cell death *in vivo* by controling the lysosomal membrane permeabilization (LMP) in mammary epithelial cells. Thus, Stat3 plays dual role in mammary epithelium, by controling the physiolgical apoptosis in normal mammary epithelium it fucntion as a pro-apoptotic molecule while protecting breast tumor cells from apoptotic stimuli it function as pro-oncogenice molecule. However, the molecular mechanism that responsible for switching of activated Stat3 from a pro-oncogenic signal to a pro-apoptotic signal during mammary gland involution is not well understood. We recently fond SLC5A8, a $Na^+$-coupled monocarboxylate transporter and tumor suppressor, could be the molecular switch that convert the pro-oncogenic function of Stat3 into pro-apoptotic signaling. Our studies have shown that SLC5A8 mediated butyrate play a major role in Stat3 associated cellular apoptosis during mammary gland involution as well in human breast cancer cells.

## Objectives

**The objective of this study is to delineate the molecular switch, which converts the STAT3 associated pro-oncogenic signaling into pro-apoptotic and tumor suppressor function in human breast cancer.**

## Results

### Slc5a8 and Stat3 expression is induced in early onset of mammary gland involution
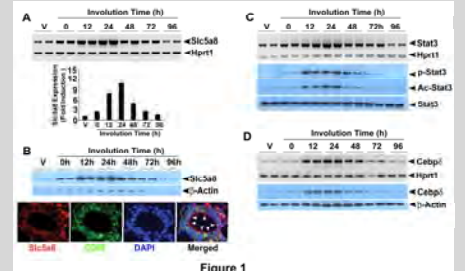


**Figure 1: Slc5a8 and Stat3 expressions are induced in early onset of mammary gland involution.** RNA and protein samples were isolated from the mammary glands of virgin (V), lactation day 10 (0h involution) and different time of involution (Day 0.5, 1, 2, 3 and 4). (A) Slac5a8 mRNA expression was analyzed by semi-quantitative and real-time PCR analyses using mouse Slc5a8 specific primers. (B) Slc5a8 protein expression was analyzed by western blot and immunohistochemical (IHC) analyses by using Slc5a8 antibody. In (IHC) CD98 was used as a basolateral membrane marker. C) Stat3 mRNA expression was analyzed by PCR analysis and Stat3 protein expression was analyzed by western blot analysis using phospho- and acetylation-specific Stat3 antibody. We also analyzed the mRNA and protein expressions C/ebpδ, a down-stream target of Stat3, as a positive marker for involution.

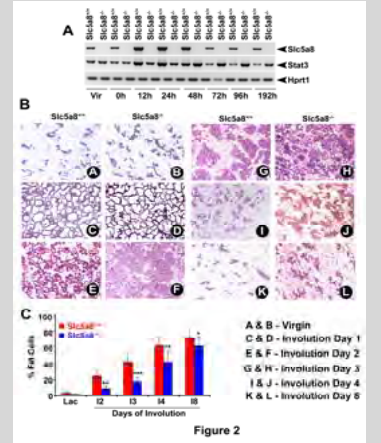### Slc5a8 knock down is associated with mammary gland involution delay



**Figure 2: Slc5a8 inactivation is associated with mammary gland involution delay.** (A) Slc5a8 and Stat3 expressions in Slc5a8 wild type (Slc5a8+/+) and knockout (Slc5a8-/-) mouse mammary glands were analyzed by semi-quantitative analysis. (B) H and E sections of Slc5a8 wild type and knockout mouse mammary glands of virgin and different time of involution. (C) Quantification fat cells in Slc5a8 wild type and knockout mouse mammary glands of virgin and different time of involution.

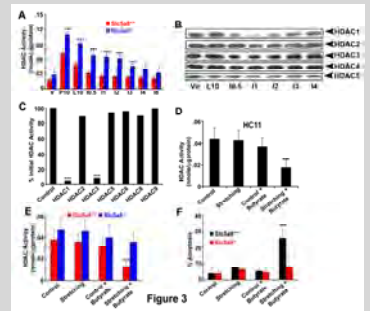### Mammary gland involution is associated with inhibition of HDAC1 and HDAC3



**Figure 3: Mammary gland involution delay is associated with inhibition of HDACs.** (A) HDAC activity was measured from the cell lysates, which prepared from the Slc5a8 WT and KO mouse mammary glands of virgin, lactation and different times of involution. (B) HDACs (HDAC1-5) protein expression of Slc5a8 WT mouse mammary glands. (C) HDAC activity was measured from the recombinant HDACs (1, 2, 3, 5, 8 and 9). (D) HDAC activity was measured from the cell lysates, which prepared from the HC11 cells, a normal mouse mammary epithelial cell line, after mechanical stretching in the presence and absence of butyrate. (E) HDAC activity was measure from the cell lysates that prepared from the Slc5a8 WT and KO mouse mammary epithelial cells after mechanical stretching with and without butyrate. (F) Apoptotic cell death was measured in the Slc5a8 WT and KO mouse mammary epithelial cells after mechanical stretching with and without butyrate.

### Mechanical stretching induced Slc5a8 and Stat3 acetylation and apoptosis in the presence of Slc5a8 substrates
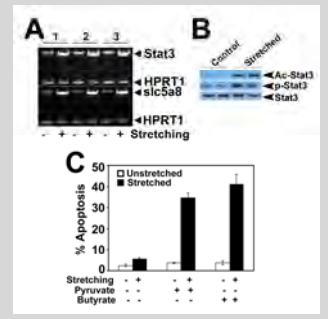


**Figure 4: Mechanical Stretching induced Slc5a8 and Stat3 expression.** RNA and protein were extracted from the HC11, a normal mouse mammary epithelial, cells that were mechanically stretched for 3h. (A) Slc5a8 and Stat3 mRNA expression was analyzed by semi-quantitative PCR. (B) Acetylation and phosphorylation status of Stat3 were tested by western blot analysis using specific antibodies. (C) Cellular apoptosis was measured in HC11 cells with and without mechanical stretching and Slc5a8 substrates pyruvate and butyrate by FACS analysis

### Mammary gland specific Slc5a8 expression and butyrate implantation induced early onset of mammary gland involution
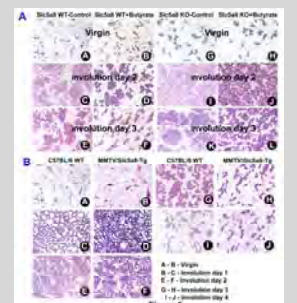


**Figure 5: Mammary gland specific Slc5a8 expression induced early onset of mammary gland involution.** (A) A continuous release of butyrate tables (10mg for 90 days) were implanted in Slc5a8 WT and KO mice and mammary glands were analyzed from virgin and different time of involution. Representative images of H & E sections of Virgin, involution day 2 and 3 are shown. (B) Mammary gland specific Slc5a8 expressing transgenic mice (MMTV/Slc5a8-Tg) were developed and the mammary glands were analyzed from virgin, lactation and different time of involution. Representative images of H & E sections of virgin, lactation day 10, involution days 1, 2, 3 and 4 are shown.

## Summary and Conclusions

- ❖ Slc5a8 and Stat3 expression are highly induced in early onset of mammary gland involution.
- ❖ Slc5a8 knockout is associated with involution delay.
- ❖ Slc5a8 activation is associated with inhibition of HDAC activity and HDAC1 and HDAC3 expression.
- ❖ Mechanical stretching also associated with activation of Slcta8 and Stat3 acetylation and phosphorylation.
- ❖ Mechanical stretching induced mammary epithelial apoptosis only in the presence of Slc5a8 substrates pyruvate and butyrate.
- ❖ Early onset of mammary gland involution occurs in the presence of functional Slc5a8 and continuous release of butyrate.
- ❖ Mammary gland specific over expression of Slc5a8 is associated with early onset of mammary gland involution and apoptosis.

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# Molecular mechanism of SLC5A8 inactivation in breast cancer

Muthusamy Thangaraju and Vadivel Ganapathy

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Breast cancer is a leading cause of death in women, but the molecular mechanism for the pathogenesis of this dreadful disease is not clearly defined. SLC5A8, a transporter of butyrate and other monocarboxylates, is silenced in more than 10 different types of cancers including breast cancer. This is the first tumor suppressor ever identified in a very broad spectrum of human malignancies. The tumor suppressor function of SLC5A8 is associated with inhibition of histone deacetylases (HDACs). The substrates of SLC5A8 (butyrate and pyruvate) are HDAC inhibitors. These HDAC inhibitors specifically induce apoptosis in tumor cells. Tumor cells inactivate *SLC5A8* at an early stage of tumor development to prevent the entry of butyrate and pyruvate into cells. In order to understand the molecular mechanisms that involved in inactivation of SLC5A8 in human breast cancer, we genetically engineered the human normal mammary epithelial cells (MCF10A) with various oncogenic stimuli and found that constitutively active H-RASG12V plays an important role in inactivation of SLC5A8 through activation of DNA methyl transferases (DNMTs), specifically DNMT1, in this cell. Using the serially transformed MCF10A-HRASG12V stable cells, we found that HRASG12V inactivates SLC5A8 expression several fold by activating DNMT1. We also found that several human breast cancer cells maintain high expression of DNMT1 as well as DNMT activity when compared to normal mammary epithelial cells and that this high DNMT1 expression is associated with SLC5A8 inactivation in the human breast cancer cells. Further, we found that either inhibition of DNMTs by 5-azacytidine or homologous deletion of DNMT1 reactivates SLC5A8 expression. To understand the tumor suppressor role of *Slc5a8*, we crossed the *Slc5a8* wild type (*Slc5a8^{+/+}*) and knockout (*Slc5a8^{-/-}*) mice with MMTV-Hras-Tg mice, a mouse model of mammary tumor, and found that deletion of *Slc5a8* is associated with an early onset of mammary tumorigenesis and promotion of lung metastasis. We have also generated a transgenic mouse with a mammary gland-specific overexpression of *Slc5a8* (MMTV-*Slc5a8*); the overexpression of Slc5a8 in mammary gland effectively protects MMTV-Hras-Tg mice from mammary tumor. The same protective effects can be elicited pharmacologically by treating MMTV-Hras-Tg mice with 5-azacytidine, which induces the expression of Slc5a8 in tumors via inhibition of DNA methylation. These results demonstrate that oncogenic HRAS plays an important role in inactivation of SLC5A8 in human breast cancer. A clear understanding of the molecular mechanisms by which HRAS activates DNMT1 and inactivates SLC5A8 will aid in the design and development of novel therapeutic strategies for the prevention and treatment of breast cancer.

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# SIRT1 is essential for oncogenic signaling by estrogen/estrogen receptor α in breast cancer

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All authors declare no conflict of interest.

**Running Title**: Promotion of breast cancer by E2-ERα occurs through SIRT1

**Key Words:** Breast cancer, Estrogen, Estrogen receptor, SIRT1, p53

**Abstract**

The NAD-dependent histone deacetylase SIRT1 is overexpressed and catalytically activated in a number of human cancers, but recent studies argue have actually suggested that it may function as a tumor suppressor and metastasis inhibitor *in vivo*. In breast cancer, SIRT1 stabilization has been suggested to contribute to the oncogenic potential of the estrogen receptor α (ERα), but SIRT1 activity has also been associated with ERα deacetylation and inactivation. In this study, we show that SIRT1 is critical for estrogen to promote breast cancer. ERα physically interacted and functionally cooperated with SIRT1 in breast cancer cells. ERα also bound to the promoter for SIRT1 and increased its transcription. SIRT1 expression induced by ERα was sufficient to activate anti-oxidant and pro-survival genes in breast cancer cells, such as catalase and glutathione peroxidase, and to inactivate tumor suppressor genes such as cyclin G2 (CCNG2) and p53. Moreover, SIRT1 inactivation eliminated estrogen/ERα-induced cell growth and tumor development, triggering apoptosis. Taken together, these results indicated that SIRT1 is required for estrogen-induced breast cancer growth. Our findings imply that the combination of SIRT1 inhibitors and anti-estrogen compounds may offer more effective treatment strategies for breast cancer.

**Introduction**

SIRT1 (Silent information regulator 1) is a NAD$^+$-dependent histone deacetylase, which is implicated in multiple biologic processes in several organisms (1, 2). All seven members of the sirtuin family (SIRT1–7) catalyze either protein deacetylation or ADP-ribosylation. SIRT1 has protein deacetylase activity, but no ADP-ribosyl transferase activity. SIRT1 targets many transcription factors, such as p53, FOXO, E2F1, NF-κB, PGC-1α, LXR, and MyoD (3, 4). Previous studies have shown that SIRT1 is overexpressed and/or catalytically activated in varieties of human cancers and that SIRT1 overexpression blocks apoptosis and senescence, and promotes cell proliferation and angiogenesis (5-8). Inhibition of SIRT1 induces growth arrest and apoptosis in a variety of cancer cells (9, 10). Further, p53 is a target for SIRT1; SIRT1 binds and deacetylates p53 resulting in its inactivation (11). In turn, activated p53 downregulates SIRT1 translation via miR-34a (12). *p53*-null mice have increased levels of SIRT1 and several *p53*-null tumor cell lines show SIRT1 overexpression (13). These studies clearly show that SIRT1 functions as an oncogene.  However, recent studies have suggested that SIRT1 may function as a tumor suppressor. SIRT1 suppresses intestinal tumorigenesis and colon cancer growth in a β-catenin-driven mouse model of colon cancer (14). *Sirt1$^{-/-}$* mice show impaired DNA damage response, evidenced by genomic instability and tumorigenesis, and activation of SIRT1 protects against mutant BRCA1-associated breast cancer (15).

Estrogen (E2) influences many physiologic processes in mammals (16, 17). E2/ERα signaling plays an important role in the regulation of mammary gland development and function, and also contributes to the onset and progression of breast cancer. More than 70% of human breast cancers express ERα, and elevated levels of ERα in benign breast epithelium correlate with

increased risk of breast cancer (18). In addition, studies have shown a positive correlation between ERα expression and age-dependent increase in cancer incidence and metastasis (19, 20).

SIRT1 is a histone deacetylase; it deacetylates several histone and non-histone proteins and thereby it inactivates tumor suppressor genes and other target proteins. ERα is one of the several targets of SIRT1. p300 acetylates ERα and the acetylation is reversed by SIRT1 (21). However, recent studies have shown that inhibition of SIRT1 suppresses ERα expression (22). Mammary gland-specific *Sirt1* deletion interferes with E2-stimulated growth signaling in normal and malignant mammary epithelial cells (23). E2 prevents age-related bone loss by inducing SIRT1 expression in the bone marrow (24). In addition, E2 recruits ERα and SIRT1 at the NQO1 (an NRF2-dependent detoxifying enzyme) promoter to inhibit transcription (25). Since ERα and SIRT1 cooperate in the development of mammary tumorigenesis, a clear understanding of the interaction at the molecular level could potentially open up new therapeutic avenues for the treatment of breast cancer.

**Materials and Methods**

*Cell lines, plasmids and transfection*

The human normal mammary epithelial cell line HMEC was obtained from the Lonza, Walkersville, MD while the other two human normal mammary epithelial cell lines, MCF10A and MCF12A, were obtained from ATCC, Manassas, VA. HBL100, also a human normal mammary epithelial cell line, was kindly provided by Dr. S. Sukumar, Johns Hopkins University, Baltimore, MD. ER-positive human breast cancer cell lines (MCF7, T47D, ZR75.1, BT474, BT483, MDA-MB361, MDA-MB415) and the ER-negative human breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468 and HCC1937) were obtained from ATCC,

Manassas, VA. The HMEC and MCF10A cells were grown in MEGM complete medium and MCF12A cells was grown in DMEM/F12 medium with 5% horse serum, 20 ng/ml human epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01mg/ml bovine insulin and 500 ng/ml hydrocortisone. HBL100 cells was grown in McCoy 5A with 10% FBS. MCF7 and BT20 cells were grown in DMEM medium with 10% FBS. T47D, ZR75.1, BT474, BT485 and HCC1937 cells were grown in RPMI 1640 medium with 10% FBS. MDA-MB-231, -361, and MDA-MB-468 cells were grown in Leibovit's L-15 medium with 10% FBS. MDA-MB415 cells was grown in Leibovit's L-15 medium with 15% FBS and 0.01mg/ml insulin.

*Plasmid constructs*: Details are given in supplementary information.

*Generation of SIRT1shRNA-expressing stable cell lines:* Details are given in supplementary information.

*Immunoprecipitation experiments*

Immunoprecipitation (IP) was accomplished with the Universal Magnetic Co-IP kit. HMEC, MCF10A, MCF7, ZR75.1, MB231 and MB453 cells' extracts were first incubated with protein A/G agarose beads. The cleared supernatants were incubated either with SIRT1-specific antibody or with ERα-specific antibody overnight before addition of protein A/G agarose beads. Normal rabbit IgG was used as control. After washing, immunoprecipitated materials were eluted and immunoblotted (IB) with human anti-SIRT1 and anti-ERα antibodies. For assessment of ERα acetylation, nuclear extracts were used for IP with an antibody specific for acetylated lysine, and the immunoprecipitates were used for immunoblotting with an ERα-specific antibody.

*Immunofluorescence:* Details are given in supplementary information.

*Chromatin immunoprecipitation (ChIP) assays*

ZR75.1 cells was transfected with expression constructs of ER family members or SIRT1-7. Chromatin immunoprecipitation (ChIP) assays were carried out using a ChIP assay kit (Millipore) using human SIRT1, ERα and mouse IgG antibodies. After ChIP, genomic DNA present in the immunoprecipitates was analyzed by PCR using the promoter-specific primers (Supplementary Table S1)

*Immunoblot analysis*

For immunoblot (IB) analysis, cell lysates were prepared by sonication of cells in cell lysis buffer with protease inhibitors. Protein samples were fractionated on SDS-PAGE gels and transferred to Protran nitrocellulose membrane (Whatman GmbH). Membranes were blocked with 5% non-fat dry milk and exposed to primary antibody at 4°C overnight followed by treatment with appropriate secondary antibody, conjugated to horseradish peroxidase at room temperature for 1 h, and developed by Enhanced Chemiluminescence SuperSignal Western System.

*RT-PCR*

SIRT1, ERα, p53, c-Myc, cyclin G2, cyclin G1, survivin and BMP7 mRNA expressions were determined by RT-PCR. Total RNA, isolated from ZR75.1-pLKO.1 and ZR75.1-SIRT1shRNA cells, was reverse transcribed using the GeneAmp RNA PCR kit (Applied Biosystems). PCR was performed on Veriti thermocycler (Applied Biosystems) using the human-specific primers (Supplementary Table S2). Representative images of triplicate experiments are shown.

SIRT1expression in kidney, lung, liver, heart and colon was analyzed by RT-PCR in male and female mice (C57BL/6) at different ages (3, 6, and 9 months). We used three animals in each age group and SIRT1 expression was quantified by densitometry.

*Lipid peroxidation, glutathione peroxidase and superoxide dismutase Assays*

Commercial kits were used to assay the lipid peroxidation product malondialdehyde (MDA), glutathione peroxidase (Gpx) and superoxide dismutase (SOD) activities as per the manufacturer's instructions. Details are given in supplementary information.

*Cell cycle analysis*

Cells were fixed in 50% ethanol, treated with 0.1% sodium citrate, 1 mg/mL RNase A, and 50 μg/mL propidium iodide, and subjected to fluorescence-activated cell sorting (FACS, Becton Dickinson) analysis.

*Colony formation assay*

ZR75.1-pLKO.1 and ZR75.1-SIRT1shRNA cells were seeded in six-well plates (10,000 cells/ well) and grown in E2-free medium. After 24 h, cells were exposed to 10 nM 17β-estradiol for 2 weeks, changing the medium every 3 days. Cells were washed with PBS and fixed in 100% methanol for 30 min followed by staining with KaryoMax Giemsa stain for 1 h. The wells were washed with water and dried overnight at room temperature. Finally, cells were lysed with 1% SDS in 0.2 N NaOH for 5 min and the absorbance of the released dye was measured at 630 nm.

*Mouse xenograft studies*

Female athymic nude mice (6- to 8-wk old) were purchased from Taconic, Hudson, NY). Animals were housed in a pathogen-free isolation facility with a light/dark cycle of 12/12 h and fed with rodent chow and water *ad libitum*. We included 6 mice in each group. Seven days before tumor induction, mice were anesthetized and 3-mm pellets containing 17β-estradiol, 0.18 mg/21-day release (Innovative Research of America), was implanted subcutaneously in the animal's back. The pellets provided a continuous release of E2 at serum concentrations of 150–250 pM, which is in the range of physiologic levels seen in mice during the estrous cycle. One

week after surgery, ZR75.1-pLKO.1 and two clones of ZR75.1-SIRT1shRNA cells ($1x10^7$ cells in 100 µl PBS) were injected subcutaneously in the mammary fat pad. Tumor volume was quantified by measuring the length and width of the tumor every 7 days for 4 weeks using a caliper.

*Collection of tissue samples from mouse*

Tissues (kidney, lung, liver, heart and colon) were harvested from 3, 6 and 9 months-old male and female mice as per the Medical College of Georgia IACUC approved protocols. Tissue samples were immediately processed for the isolation RNA and protein samples.

*Collection of ER-positive and ER-negative mammary tissues*

The primary breast cancer specimens and corresponding normal control specimens, adjacent to the tumor, were obtained from the Medical College of Georgia Tumor Bank, which is operated jointly by the MCG Cancer Center and the Department of Pathology. The Tumor Bank has the approval from the Institutional Review Board and Human Assurance Committee for the collection of the tissues. The Tumor Bank has determined the tumor grade and the molecular signature of each of the cancer specimens in terms of expression of ER and progesterone receptor (PR) and also whether or not the tumor overexpresses HER2. We obtained 12 specimens and the corresponding normal controls from the Tumor Bank. In addition, we had 4 specimens from a commercial source. RNA was extracted from 12 ER-positive and 4 ER-negative normal and breast cancer tissues using the TRIZOL reagent (Invitrogen).

*Institutional compliance*

The animal experiments reported in this study were approved by the Medical College of Georgia IACUC and Biosafety Committees. Similarly, human breast cancer tissue and the surrounding

normal tissues were obtained from the Medical College of Georgia tumor bank as per the approval of the Institutional Review Board and Human Assurance Committee.

## *Statistical analyses*

Statistical analysis was done using one-way ANOVA followed by Bonferroni multiple comparison test. The software used was Graph Pad Prism, version 5.0. A p value <0.05 was considered statistically significant.

## Results

**Functional cooperation between E2/ERα and SIRT1:** To understand the functional association between E2/ERα and SIRT1 in breast cancer, first we compared the expression levels of ERα and SIRT1 in ER-positive and ER-negative normal and breast tumor tissues as well as in nonmalignant and malignant mammary cell lines. We found a significant positive correlation between ERα and SIRT1 expressions (Fig. 1A, B and C). ERα-positive tumors and cancer cell lines expressed high levels of SIRT1 than ERα-negative tumors and cancer cell lines. Similarly, both ERα and SIRT1 proteins are overexpressed in breast tumor tissues compared to normal mammary tissues (Fig. S1). To confirm this positive association further, we treated ZR75.1 cells with E2 in the presence and absence of antiestrogens and monitored the expression levels of SIRT1. E2 significantly induced SIRT1 expression, and the effect was markedly blunted in the presence of antiestrogens (Fig. 1D). Similarly, when ZR75.1 cells were passaged several times in estrogen-free culture medium, ERα disappeared in these cells as did SIRT1 at passage numbers greater than 10 (Fig. 1E). These data showed that ligand-occupied ERα induces SIRT1 expression in mammary epithelial cells. This was supported further by the findings that SIRT1 levels were higher in various tissues in female mice than in male mice (Fig. S2).

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SIRT1expression also correlated positively with E2 in various stages of mammary gland development (data not shown). All these results suggest that E2 plays a prominent role in regulation of SIRT1 expression.

**ERα binds to SIRT1 promoter and forms a complex in human breast cancer cells:** Unequivocal evidence for the induction of SIRT1 expression by E2/ERα came from the analysis of SIRT1 promoter activity. Analysis of the human SIRT1 promoter sequence revealed the presence of five putative ERα binding sequences in the upstream regulatory region (Fig. 2A). We cloned the 2.2 kb human SIRT1 promoter and subcloned it into pEGFP and luciferase (Luc) reporter vectors. These constructs were used for transfection into ERα-positive ZR75.1 cells. E2 treatment induced the SIRT1 promoter-specific EGFP fluorescence (Fig. 2B) as well as SIRT1 promoter-specific luciferase activity (Fig. 2C). Estrogen-induced EGFP fluorescence and luciferase activity were markedly blunted in the presence of antiestrogens (Fig. 2B and C). These experiments were complemented with ChIP assay. We expressed ER (ERα and ERβ) and ERR (ERRα, ERRβ and ERRγ) family members in ZR75.1 cells, performed immunoprecipitation with antibodies specific for these proteins, and examined the SIRT1 promoter in the immune complex by PCR. These studies showed that both ERα and ERRα interact with SIRT1 promoter and form a complex (Fig. 2D). These studies clearly demonstrate the transcriptional regulation of SIRT1 by E2 in mammary epithelial cells.

**Interaction of SIRT1 with ERα:** Then, we wanted to test whether ERα and SIRT1 physically interact and functionally colocalize with each other in mammary epithelial cells. We performed a series of co-immunoprecipitation assays using ERα-positive and ERα-negative cell lines. The interaction between SIRT1 and ERα was clearly evident in MCF7 and ZR75.1 cells that express

both proteins (Fig. 3A). In these cells, both SIRT1 and ERα were colocalized in the nucleus (Fig. 3B). This interaction and nuclear colocalization was also confirmed in ERα-negative cells (MCF10A and MB231) following ectopic expression of ERα (Fig. 3C, D). There are two estrogen receptors, ERα and ERβ, and three estrogen receptor-related proteins, ERRα, ERRβ, and ERRγ; all these proteins share significant sequence homology (26). Except ERβ, all other members are associated with breast cancer initiation and progression (27). Therefore, we examined the interaction of SIRT1 with both ERs and ERRs in ZR75.1 cells that endogenously express all these proteins. These studies showed that SIRT1 interacts not only with ERα but also with ERRα (Fig. S3A and B). This was further confirmed in MCF7 cells as well as in two ER-negative human mammary epithelial cell lines, HMEC and HBL100 (data not shown). To test whether ERα specifically interacts with SIRT1 or also with other sirtuins, we expressed Flag-tagged SIRT1-7 in ZR75.1 cells, and examined the presence of ERα in immunoprecipitates with an anti-Flag antibody. The interaction was evident only with SIRT1 (Fig. S3C).

**ERα ligands and catalytically active SIRT1 are required for the interaction between ERα and SIRT1:** In the classical mechanism, E2 binds to ERα and the resultant complex interacts with the estrogen-response elements in E2 target genes, consequently promoting their transcription. To examine whether the interaction between ERα and SIRT1 requires E2 and whether functional disruption of E2/ERα complex with antiestorgens affects the interaction, we cultured ZR75.1 cells in E2-free medium and then treated the cells with E2 and antiestrogens. Immunoprecipitation experiments using these cell lysates showed that SIRT1-ERα interaction requires E2 and that functional disruption of E2/ERα complex dramatically reduces the interaction (Fig. 4A). Similarly, inhibition of the catalytic activity of SIRT1 with SIRT1

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inhibitors also disrupted the interaction (Fig. 4A). In contrast, inhibition of type I and type II HDACs did not affect the interaction between ERα and SIRT1. This suggests that active ERα as well as catalytically active SIRT1 is required for the interaction. Further, the requirement of a catalytically active SIRT1 suggests that the interaction between ERα and SIRT1 may be regulated by SIRT1-mediated deacetylation of ERα. Therefore, we examined the acetylation status of ERα in the presence (SIRT1 overexpression) and absence (SIRT1 inhibitors) of SIRT1. Neither SIRT1 overexpression nor inhibition influenced the acetylation status of ERα (Fig. 4B), showing that SIRT1 is not involved in the deacetylation of ERα. To confirm this observation further, we used SIRT1shRNA in ZR75.1 cells (ERα-positive cells with endogenous SIRT1) and MDA-MB453 (ERα-negative cells with endogenous SIRT1) and monitored the protein expression and acetylation status of ERα (Fig. 4C, data not shown). Silencing of SIRT1 did not change either the expression or the acetylation status of ERα. As a positive control, we monitored the acetylation of p53, a known target for SIRT1. As expected, silencing of SIRT1 increased p53 acetylation (Fig. 4D). A recent study has shown that inhibition of SIRT1 suppresses ERα expression in breast cancer cells, suggesting that SIRT1 might be involved in the regulation of ERα expression (22). To confirm these observations, we treated ERα-positive breast cancer cells with SIRT1 inhibitors and analyzed ERα expression. Treatment of ERα-positive breast cancer cells with relatively high concentrations of sirtinol decreased ERα expression, and this process was accompanied with an increase in apoptosis (Fig. S4A and C). In contrast, another SIRT1 inhibitor, nicotinamide, neither affected ERα expression nor induced apoptosis in these three cell lines (Fig. S4B and D). These results suggest that sirtinol-associated ERα downregulation may not be directly related to SIRT1 inhibition.

**SIRT1 is required for activation of antioxidant enzymes by E2-ERα in breast cancer cells:**

All the above results show that E2/ERα induces the transcriptional activation of SIRT1 and stabilizes SIRT1 expression by protein-protein interaction and that functional disruption of E2/ERα reduces SIRT1 expression. Thus, we wanted to find out the functional consequences of E2/ERα and SIRT1 interaction in breast cancer. It is known that E2 prevents reactive oxygen species (ROS) formation and thus protects cells from DNA damage. A well known example of E2-associated protection against ROS formation is that mitochondria from females produce approximately half the amount of ROS compared to males, and females express higher levels of antioxidative enzymes, especially Mn-SOD and Gpx, than males (28). Thus, females have a natural protection against ROS-induced cell damage. E2 also protects tumor cells from ROS-induced cell death by activating these enzymes. Further, recent studies have shown that overexpression of SIRT1 increases the transcription of SOD in human hepatoma cancer cell lines (29). This suggests that both E2/ERα and SIRT1 upregulate antioxidant enzymes to promote cell survival. Therefore, we examined the role of SIRT1 in the activation of the antioxidant machinery by E2. First we compared the extent of DNA damage, with or without UV radiation, in control (pLKO.1) and SIRT1-knockdown (SIRT1 shRNA) ZR75.1 cells in the presence and absence of E2. E2 prevented DNA damage in control cells to a significant extent, but did not do so in SIRT1 knockdown cells (Fig. 5A). The E2-mediated cellular defense was also reflected in the extent of cellular apoptosis (Fig. 5B), production of malondialdehyde (a surrogate marker for ROS) (Fig. 5C), and the expression and activities of the antioxidant enzymes Mn-SOD and Gpx (Fig. 5D, E and F); but the protective effect of E2 was evident only in control cells and not in SIRT1 knockdown cells. These studies showed that SIRT1 is required for the E2-mediated cellular defense mechanism in human breast cancer cells.

**SIRT1 is required for the suppression of p53 by E2-ERα:** Studies have shown that ERα directly binds to p53 and represses its transcription and thus it interferes with p53-mediated cell cycle arrest and apoptosis (30). SIRT1 also binds and deacetylates p53 and inactivates its function. Thus, p53 is a common target for both ERα and SIRT1; therefore we determined whether SIRT1 is required for the inactivation of p53 by E2/ERα complex. We first monitored the transcriptional activity of E2/ERα by examining the expression levels of various E2/ERα target genes in control (pLKO.1) and in SIRT1 knockdown (shRNA) cell lines. The induction of c-Myc, cyclin D1 and BMP7 by E2/ERα was independent of SIRT1, but the downregulation of p53 and cyclin G2, and the induction of survivin by E2/ERα was dependent on SIRT1 (Fig. 6A). Because E2 treatment significantly reduced p53 mRNA expression in the pLKO.1 stable cell line and SIRT1 knockdown abolished that effect and thus, we wanted to examine the role E2 and SIRT1 in the transcriptional regulation of p53 at the promoter level. Genomic database analysis of p53 promoter suggested that it has 10 consensus binding sites for ERα (Fig. S5A). We transfected ZR75.1-pLKO.1 and ZR75.1-SIRT1shRNA cells with 2.4 kb and 356-bp p53 promoter-luciferase reporter plasmids (kindly provided by Dr. S. Sukumar, Johns Hopkins University) and treated the cells with E2 for 24 h. We observed that E2 treatment significantly suppressed the p53-promoter (2.4 kb)-dependent reporter activity only in ZR75.1-pLKO.1 cells (Fig. S5B). E2 treatment did not affect the shorter p53-promoter (356-bp) in these cells. Interestingly, SIRT1 knockdown itself transactivated the p53-promoter several-fold, but E2 treatment did not affect the p53-promoter-dependent reporter activity in these cells (Fig. S5B). These results suggest that SIRT1 is required for the transcriptional suppression of p53 by E2 in breast cancer cells.

The E2-induced p53 suppression in ZR75.1-pLKO.1 cells was not observed at the protein level even though the promoter activity showed suppression (Fig. 6B). This suggests that E2-mediated p53 inactivation is associated with post-translational modification and that SIRT1 might play role in this event. Therefore, we examined the role of SIRT1 in the acetylation of p53 and cyclin G2. In control cells, E2 caused deacetylation of p53 and cyclin G2, but this effect was abolished in SIRT1 knockdown cells (Fig. 6B). ChIP assays with ZR75.1 cells transfected with ER and ERR family members showed that immunoprecipitates with anti-SIRT1 antibody contained the promoters of p53 and cyclin G2 only in cells expressing ERα and ERRα (Fig. 6C). Similarly, ChIP assays with cells transfected with SIRT1-7 showed that immunoprecipitates with anti-ERα antibody contained the promoters of p53 and cyclin G2 only in cells expressing SIRT1. This was not the case for the other E2/ERα target genes c-Myc, cyclin D1, survivin, and BMP7. These results suggest that E2 recruits ERα and SIRT1 to suppress p53 and cyclin G2 expression in ER-positive breast cancer cells.

**Loss of SIRT1 is associated with elimination of E2-induced cell survival and mammary tumorigenesis:** Since E2 promotes the development and progression of many hormone-dependent cancers, especially breast cancer, and SIRT1 also promotes several human malignancies by deacetylating tumor suppressor genes, we wanted to test whether SIRT1 plays a role in E2-induced mammary tumorigenesis. To determine the role of SIRT1 in E2-mediated promotion of cell survival and breast cancer growth, we first monitored the ability of E2 to promote colony formation *in vitro* in control cells and SIRT1 knockdown cells. E2 was able to induce colony formation only in control cells but not in SIRT1-depleted cells (Fig. 7A). This phenomenon was confirmed *in vivo* in mouse xenografts. Xenografts with control ZR75.1 cells

grew in a time-dependent manner, and the growth was promoted by E2 (Fig. 7B). In contrast, SIRT1-depleted ZR75.1 cells failed to grow in xenografts with or without E2 (Fig. 7B), showing that SIRT1 is absolutely required for the promotion of tumor growth by E2. To confirm the functional cooperation between ERα and SIRT1 in the promotion of the growth of xenografted breast cancer cells, we treated the three different human breast cancer cell lines (MCF7, T47D and ZR75.1) with Tamoxifen (an antiestrogen) and Sirtinol and nicotinamide (SIRT1 inhibitors) alone or in combination. The combination treatment produced a greater magnitude of apoptosis in these cells than the individual treatment (Fig. S6). This was true even for the Tamoxifen-resistant cell line MCF7-TAM(R) (Fig. S7A). Interestingly, the MCF7-TAM(R) cells had higher levels of SIRT1 expression than the parent MCF7 cells (Fig. S7B). We confirmed this further with SIRT1 knockdown in ZR75.1 cells where antiestrogens caused more apoptosis in SIRT1 knockdown cells than in control cells (Fig. 7C). Based on these results, we propose a model for the interaction of ERα and SIRT1 and a molecular mechanism involved in the promotion of breast cancer by the ERα-SIRT1 complex (Fig. 7D).

**Discussion**

The association of estrogen and its receptor (ERα) in development and progression of many hormone-dependent cancers, including breast cancer, has been well established. In the absence of estrogen, ERα is inactivated by the binding of a heat shock protein (HSP90) within the nucleus. Upon estrogen binding, the receptor undergoes conformational change so that HSP90 is released, enabling ERα to activate gene transcription. ERα functions as a transcriptional activator by binding to the estrogen response element in the target gene promoter (16, 17). ERα also functions as a transcription repressor upon binding to certain antagonists, such as tamoxifen, as

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well as to various corepressors and histone deacetylases (31). Thus, depending on the binding partner, ERα functions as either transcriptional activator or repressor of the target gene. Our findings demonstrate for the first time that SIRT1 is the binding partner of ERα in mammary epithelial cells and that the ERα-SIRT1 complex functions as a transcriptional activator of Mn-SOD and Gpx and as a transcriptional repressor of p53 and cyclin G2. In addition to its role as a binding partner of SIRT1, ERα also regulates the expression of SIRT1 through transcription as well as by protein-protein interaction. Our results also suggest that SIRT1 could be a downstream target of E2 in mammary epithelium.

In our study, we found a direct physical interaction between ERα and SIRT1. This interaction requires a catalytically active SIRT1. However, SIRT1 does not affect the acetylation status of ERα. Furthermore, knockdown of SIRT1 in ER-negative breast cancer cells is not able reactivate ERα expression. However, HDAC1 and HDAC3 deacetylate ERα and knockdown of these HDACs reactivates ERα expression (Fig. S8). This suggests that SIRT1 does not play a role in deacetylation of ERα. E2/ERα induces SIRT1 expression by directly binding to SIRT1 promoter. We conclude that E2/ERα signaling activates SIRT1 and that SIRT1 is the molecular transducer of E2-induced oncogenic signaling in mammary epithelial cells. Further, we also found a direct interaction between SIRT1 and ERRα in human breast cancer, and ER-negative breast cancer cells still have high expression of SIRT1 compared to normal mammary epithelial cells. In addition, we found higher levels of ERRα expression in ER-negative breast cancer cells than in normal and ER-positive breast cancer cells (unpublished data). These observations suggest that SIRT1 and ERRα might play a crucial role in regulation of survival signaling in ER-negative breast cancer. The findings in the literature that ERRα is critical for the growth of ER-

negative breast cancer and that SIRT1 directly interacts with ERRα and enhances its transcriptional function (32, 33) support our data and conclusions.

E2 increases cell survival and oncogenic transformation via activation of anti-oxidative enzymes, MAPK, PI3K and telomerase, and inhibition of p53. Likewise, SIRT1 also increases cell survival and extents cellular longevity by preventing ROS formation, regulating MAPK and inhibiting p53 function. Our results demonstrate that E2 efficiently protects breast cancer cells from oxidative and radiation-induced DNA damage by inducing SOD and Gpx activity. However, E2-associated cellular protection and antioxidant enzyme induction obligatorily depends on functional SIRT1. Thus, both E2/ERα and SIRT1 target a common molecular signaling pathway to protect cells from oxidative and radiation-induced DNA damage. Our results show that ERα and SIRT1 interact with each other and promote cell survival and tumor growth in breast cancer cells.

Our studies also demonstrate that ERα binds to p53 promoter and suppresses its expression and that the process is obligatorily dependent on SIRT1. Thus, ERα-SIRT1 complex functions as a suppressor of p53 gene in breast cancer cells. Further, E2 treatment results in deacetylation of p53, and SIRT1 knockdown abolishes this effect, suggesting that SIRT1 is necessary for the ability of ERα to suppress p53 signaling in breast cancer. Similarly, E2/ERα directly binds to cyclin G2 promoter and suppresses its expression (34). Our studies show for the first time that SIRT1 is needed for this effect.

E2/ERα complex is linked to the development and promotion of breast cancer. There is also strong evidence that SIRT1 is involved in oncogenic signaling in mammary epithelial cells. First,

SIRT1 knockout mice exhibit p53 hyperacetylation and increased radiation-induced apoptosis consistent with SIRT1 inhibition of p53 function (35). This raises the possibility that SIRT1 can facilitate tumor growth by antagonizing the p53 function. Second, DBC1 (deleted in breast cancer) inhibits SIRT1 in human mammary epithelial cells, and repression of SIRT1 by DBC1 hyperacetylates and activates p53 (6, 36). Third, HIC1 (hypermethylated in cancer) binds to SIRT1 promoter and represses its transcription; at the same time, HIC1 promotes p53 expression (7). Since HIC1 is silenced in many types of tumors, including breast cancer, this results in upregulation of SIRT1, thus promoting tumorigenesis. Further, upregulation of SIRT1 has been observed in various cancers (5-8). All these findings show that SIRT1 is a tumor promoter. Our studies demonstrate unequivocally that SIRT1 plays a critical role as a tumor promoter in ER-positive breast cancer. In normal cells, the ERα-SIRT1 complex mediates several beneficial functions such as the protection of the cells from the ROS-induced DNA damage, maintainance of genomic integrity and telomerase function, and inactivation of apoptotic signaling. The same functions promote the survival and growth of tumor cells. Furthermore, SIRT1 also seems to play a crucial role in breast cancer cells in the development of resistance to anti-estrogen therapy. These findings suggest that appropriate combination therapies targeting both ERα and SIRT1 could offer a novel and effective strategy for treatment of breast cancer.

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**Figure Legends**

**Fig.1. Functional cooperation between E2-ERα and SIRT1.** (**A**) Expression of ERα and SIRT1 mRNAs in ER-positive (ER[+]) breast tumors tissues and adjacent normal tissues as well as in ER-negative (ER[-]) breast tumor tissues and corresponding normal tissues. (B) ERα and SIRT1 mRNA levels were quantified by densitometry. ** $p < 0.01$. (**C**) SIRT1 mRNA expression in human normal, ER[+] and ER[-] breast cancer cell lines. (**D**) SIRT1 mRNA expression in ZR75.1 cells, treated with or without E2 alone or in combination with TAM and ICI182740. (**E**) ERα and SIRT1 mRNA levels in ZR75.1 cells, cultured for several passages in regular and E2-free medium.

**Fig.2. ERα binds to SIRT1 promoter and forms a complex.** (**A**) SIRT1-pGL3 and SIRT1-pU3R2EGFP reporter constructs and potential ERα binding sites. (**B**) ZR75.1 cells were transfected with SIRT1-pU3R2EGFP reporter construct and then cultured in E2-free medium for 24 h. Cells were then treated with and without 17β-estradiol (E2), tamoxifen (TAM), 4-hydroxy tamoxifen (4-OH TAM) and ICI182780 for 24 h and the expression of GFP was monitored by epifluorescence. (**C**) ZR75.1 cells were transfected with SIRT1-pGL3 reporter construct and then cultured in E2-free medium for 24 h. Cells were then treated with and without E2, TAM, 4-OH TAM and ICI182780 for 24 h and luciferase activity was measured using the cell lysates. (**D**) ZR75.1 cells were transfected with both ER (ERα and ERβ) and ERR (ERRα, ERRβ and ERRγ)

family members and Chromatin immunoprecipitation (ChIP) assays were carried out using antibodies specific for these proteins. Genomic DNA present in the immunoprecipitates was examined using the SIRT1 promoter-specific primers by PCR.

**Fig.3. SIRT1 interacts with ERα.** (**A**) SIRT1 was immunoprecipitated (IP) from ER$^-$ (HMEC, MCF10A, MB231 and MB453) and ER$^+$ (MCF7 and ZR75.1) cells using SIRT1 or ERα antibodies. Immunoblotting (IB) was done with SIRT1 or ERα antibodies. (**B**) Colocalization of SIRT1 and ERα in MCF-7 and ZR75.1 cells. Hoechst staining was used to locate the nucleus. The scale bars represent 20 µm. (**C**) ER$^-$ MCF10A and MB231 cells were transfected with an expression construct of ERα. IP and IB were performed 48 h post-transfection with SIRT1 and ERα antibodies. (**D**) MCF10A cells were transfected with an ERα expression construct, and colocalization (arrows) was monitored with SIRT1 and ERα antibodies.

**Fig.4. SIRT1 interaction requires an active E2-ERα complex.** (**A**) ZR75.1 cells, treated with or without E2 (10 nM), E2 antagonists TAM (1µM), 4-OH TAM (1µM) and ICI182740 (1µM), SIRT1 inhibitors (NAD, 2 mM) and Sirtinol, 25 µM) or Type I and II HDAC inhibitor (TSA, 1 µM) were subjected to IP with an ERα antibody. The immunoprecipitates were used for IB with SIRT1 antibody. (**B**) ZR75.1 cells were transfected with pcDNA, SIRT1 and p300 expression constructs or treated with NAD$^+$, Sirtinol and TSA. Nuclear extracts from these cells were used for IP with acetyl-lysine (Ac-Lys) antibody and the immunoprecipitates were immunoblotted with an ERα antibody. (**C**) ERα expression was analyzed in control and SIRT1 knockdown ZR75.1 cells. (**D**) Nuclear extracts from control and SIRT1 knockdown ZR75.1 cells were subjected to IP with Ac-Lys antibody. The immunoprecipitates were then used for immunoblotting with ERα and p53 antibodies.

**Fig.5. SIRT1 is required for activation of antioxidant enzymes by E2/ERα.** (**A**) DNA damage, with or without UV radiation, was quantified in control and SIRT1 knockdown ZR75.1 cells with or without E2 treatment. *** p<0.001; *p<0.05. (**B**) Cell cycle analysis was performed with cells described above, and the sub G0/1 cells were quantified using cell quest software. *** p<0.001. (**C**) The levels of malondialdehyde (MDA) were measured as a surrogate for lipid peroxidation in control and SIRT1 knockdown ZR75.1 cells with or without E2 treatment. *** p<0.001; *p<0.05. (**D**) Glutathione peroxidase (Gpx) and (**E**) superoxide dismutase (SOD) activities were measured in cell lysates. ***p<0.001; ** p<0.01; *p<0.05. (**F**) Gpx and SOD mRNA levels were analyzed in control and SIRT1 knockdown ZR75.1 cells.

**Fig.6. SIRT1 is required for suppression of p53 by E2/ERα.** (**A**) The expression levels of E2 target genes (c-Myc, cyclin D1, BMP7, survivin, p53 and cyclin G2) were analyzed by RT-PCR and western blot in control and SIRT1 knockdown ZR75.1 cells with or without E2 treatment. The red highlights identify the genes whose regulation by E2 is mediated through SIRT1. (**B**) The acetylation status of p53 and cyclin G2 was analyzed in control and SIRT1 knockdown ZR75.1 cells with or without E2 treatment. (**C**) ChIP assay was performed to examine the binding of ERα/SIRT1 to E2 target genes.

**Fig.7. Loss of SIRT1 is associated with elimination of E2-induced cell survival and mammary tumorigenesis**. (**A**) Control and SIRT1 knockdown ZR75.1 cells were treated with or without E2 for 2 weeks and the resulting colonies were quantified with Giemsa staining. (**B**) Seven days before tumor induction, female athymic nude mice (6 animals per group; 3 groups) were anesthetized and 3-mm pellets containing E2, 0.18 mg/21-day release, were implanted subcutaneously in the animal's back. Another 18 animals were used as a control, without E2 treatment. One week after pellet implantation, ZR75.1-pLKO.1 and ZR75.1-SIRT1shRNA (two

independent shRNA clones shRNA#2 and shRNA#3) cells ($1x10^7$ cells in 100 µl PBS) were injected s.c. in the mammary fat pad of all animals. Tumor volume was used as a measure of tumor growth at 1, 2, 3 and 4 weeks after cell injections. We compared the tumor volume of pLKO.1 and SIRT1shRNA-induced tumor in the presence and absence of E2 and the statistical significance was calculated as ***$p<0.001$; ** $p<0.01$; *$p<0.05$*$p<0.05$ in each time point (**C**) Control and SIRT1 knockdown ZR75.1 cells were treated with E2, TAM, 4-OH TAM and ICI182780 for 48 h. Apoptotic cell death was analyzed by FACS. We compared the apoptotic cell death in control pLKO.1 and SIRT1shRNA cells treated with E2, TAM, 4-OH TAM and the statistical significance was calculated as ***$p<0.001$ (**D**). Proposed mechanism of E2-ERα and SIRT1 complex in regulation of tumor cell immortalization and tumor development.

**A**

ERα-Positive       ERα-Negaitive

N T N T N T N T N T N T N T N T N T N T N T N T N T N T N T    N T N T N T N T N T N T

◄ERα
◄SIRT1
◄GAPDH

**B**

ERα

ER α/GAPDH ratio

□ Normal   ■ Tumor

ER⁺    ER⁻

SIRT1

ER α/GAPDH ratio

■ Normal   ▨ Tumor

ER⁺    ER⁻

**C**

HMEC HBL100 MCF10A MCF12A MCF7 T47D ZR75.1 BT474 BT485 MB415 MB361 MB231 MB453 MB468 BT20 HCC1937

◄ERα
◄GAPDH
◄SIRT1
◄GAPDH

Normal    ER⁺-breast cancer    ER⁻-breast cancer

**D**

0 12 24 48   0 12 24 48   0 12 24 48   Time (h)

◄SIRT1
◄GAPDH

E2    E2+TAM    E2+ICI 182780

**E**

1   2   3   4   5   10   15   Passage #
+ − + − + − + − + − + − + −   E2

◄ERα
◄SIRT1
◄GAPDH

**Figure 1**

**A**

-2.2kb  -340 -196 -187 -153 -136  ATG  pU³R²EGFP

🔴 Potential ERα binding sites

-2.2kb  -340 -196 -187 -153 -136  ATG  pGL3-Luc

**B**

-E2 | +E2

-TAM | +TAM | +E2 -TAM | +E2 +TAM

-4OHT | +4OHT | +E2 -4OHT | +E2 +4OHT

-ICI 182980 | +ICI 182980 | +E2 -ICI 182980 | +E2 +ICI 182980

SIRT1-pU²R³-EGFP-promoter

**C**

SIRT1 Promoter Acitivity x 10³

-E2
+E2
E2 -TAM
E2 +TAM
E2 -4 -OH TAM
E2 +4-OH TAM
E2 -ICI182980
E2 +ICI182980

**D**

M  Input  No Abs  ERα  ERβ  ERRα  ERRβ  ERRγ

◄SIRT1

**Figure 2**

**A**

| | Input | Control IgG | HMEC | MCF10A | MCF7 | ZR75.1 | MB231 | MB453 | |
|---|---|---|---|---|---|---|---|---|---|
| IB: ERα | | | | | | | | | IP: SIRT1 |
| IB: SIRT1 | | | | | | | | | |
| IB: SIRT1 | | | | | | | | | IP: ERα |
| IB: ERα | | | | | | | | | |

**C**

| | Input | Control IgG | pIRES2 | ERα-pIRES2 | pIRES2 | ERα-pIRES2 | |
|---|---|---|---|---|---|---|---|
| IB: ERα | | | | | | | IP: SIRT1 |
| IB: SIRT1 | | | | | | | |
| IB: SIRT1 | | | | | | | IP: ERα |
| IB: ERα | | | | | | | |

MCF10A        MB231

Figure 3

**B**

SIRT1        ERα        SIRT1+ERα        Hoechst 33342

MCF7

ZR75.1

**D**

MCF10A (pIRES2)

MCF10A (ERα-pIRES2)

**A**

IP: ERα

IB:SIRT1

IB: ERα

Lanes: Input, Control IgG, E2, +E2, E2+TAM, E2+4-OH TAM, E2+ICI182780, Sirtinol, NAD, TSA

**B**

◄IP: Ac-Lys; IB: ERα

◄IP: ERα; IB: ERα

Lanes: Input, Control IgG, pcDNA, SIRT1cDNA, Sirtinol, NAD, TSA, p300

**C**

pLKO1, shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 | pLKO1, shRNA1, shRNA2, shRNA3, shRNA4, shRNA5

◄ SIRT1

◄ ERα

◄ β-Actin

mRNA                Protein

**D**

◄ IP: Ac-Lys
◄ IB: ERα

◄ IB: p53

◄ IB: Ac-Lys

Lanes: Input, Control IgG, pLKO1, shRNA1, shRNA2, shRNA3, shRNA4, shRNA5

**Figure 4**

Figure 5

**Figure 6**

**Figure 7**